SEP 4 1917
UNIV. OF MICH.
LIBRARY

THE JOURNAL

OF

EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D.

VOLUME XXVI, NO. 3 SEPTEMBER 1, 1917

PUBLISHED MONTHLY

AT 2419-21 GREENMOUNT AVE., BALTIMORE, MD.

BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

Entered as second-class matter at the Post Office at Baltimore, Md., under the Act of March 3, 1879

COPYRIGHT, 1917, BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

WAVERLY PRESS
THE WILLIAMS & WILKINS COMPANY
BALTIMORE, U. S. A.

HUMAN PULMONARY DISTOMIASIS CAUSED BY PARAGONIMUS WESTERMANNI.

By KOAN NAKAGAWA, M.D.

(From the Government Hospital, Shinchiku, Formosa.)

Plates 22 to 31.

(Received for publication, July 27, 1916.)

INTRODUCTION.

Pulmonary distomiasis is caused by *Paragonimus westermanni* Kerbert, and prevails extensively in the Far East. Ringer (1) discovered the parasite in 1879 in a patient at Tamsui in Formosa, and it has since been found in various parts of Japan by several observers. In certain localities it prevails as an endemic disease, and has for a long time aroused the interest of Japanese investigators. The development of the worm has, however, remained unknown except for the fact that the eggs are ejected with the sputum and that they hatch in water into free miracidia.

Prevalence of Pulmonary Distomiasis in Formosa.

Although Ringer first found cases of pulmonary distomiasis in Formosa in 1879, the actual prevalence of the disease has remained unknown. In 1910 Nagano (2) reported that the disease prevailed in the northern part of Formosa around the Prefecture of Shinchiku. During 1913 and 1914 (3), I secured records of 1,249 cases, of which 922 occurred in the Prefecture of Shinchiku, the most thickly infected region on the island. I examined the pupils of all the public schools in the Prefecture of Shinchiku in December, 1914, and found that 4.3 per cent were suffering from the disease.

The morbidity among the young is less than among adults. However, if we assume this percentage to be that of the whole population of the Prefecture of Shinchiku, there are 13,000 cases in this region alone. The results of my observation differ from those of

Matsuo and Yokokawa (4) regarding the morbidity in Sansaka, Koryo, Jukirin, and Nansho. The discrepancy is probably due to the fact that the people of those villages have sunk wells and stopped drinking the river water during the period between their investigation and mine. The infection is more prevalent among people living along large rivers, such as the Hozankei, Komodenkei, Chukokei, and Koryokei. The inhabitants of the mountainous regions also were thought to suffer from this disease, but no investigation had been undertaken. In 1914, appointed by the Commission of Investigation of Formosan Endemic Diseases, I went into the districts inhabited by savage tribes and found that in the lowlands an enormous number of cases, *i.e.*, 50 per cent of the total population, was infected; in the highlands the cases seem to be less in number, although I was unable to carry out a thorough investigation there.

Investigation of the Intermediate Hosts of Paragonimus westermanni.

The Prefecture of Shinchiku and the villages of the savages in that vicinity are favorable districts for carrying out observations upon the parasites. As soon as I was appointed the Physician-in-Chief of the Government Hospital at Shinchiku, I began to search for the intermediate hosts.

As the first step the development of the eggs and the processes of hatching were studied, but efforts at experimental infection of animals both *per os* and subcutaneously with the miracidia were unsuccessful. Hence I concluded that at least one intermediate host functions prior to the infection of man. Various species of mollusks occurring in the local streams were examined microscopically, and seventeen different kinds of cercariæ were found, but it could not be determined which represented those of *Paragonimus westermanni.*

The next step was to ascertain which mollusks the miracidia of pulmonary distomas infest. For this purpose various kinds of fresh water mollusks were kept in water in which the miracidia had been made to hatch; and it was soon found that the miracidia prefer *Melania libertina* and *Melania obliquegranosa.* Attempts to keep the mollusks alive in aquaria failed. From the fact that the only species of fresh water mollusks that live in the thickly infected regions

is *Melania libertina* Gould, it was assumed that the cercariæ with a characteristic organ in the oral sucker[1] found in the snail must be the young of the pulmonary distomas. But how the miracidia find their way into the mollusks and develop into cercariæ has not as yet been ascertained.

Because of the difficulty of verifying this assumption experimentally an effort was made to discover the second intermediate host according to Kobayashi's (5) method employed in human liver distomiasis. This consists in infecting animals with the young distomas occurring in certain fish. After many attempts I found in Kalapai, in September, 1914, many encysted cercariæ in the liver and gills of certain crabs, which bore a striking resemblance to mature pulmonary distomas. I finally succeeded in discovering their path of penetration to the lungs of the final host. The facts that the cercariæ mentioned above enter the second intermediate hosts, *i.e.*, the crab, and that the cercariæ embedded in *Melania libertina* are the cercariæ of *Paragonimus westermanni*, have now been satisfactorily established.

Development of the Worm within the Egg.

Observations on the development of the worm within the egg have already been made by Nakahama, Manson, and Garrison and Leynes, in Japan, China, and the Philippines. The procedure that I employed for the Formosan form is as follows: A quantity of expectorated sputum containing the eggs is placed in a glass dish which is filled with water to the depth of 20 to 30 mm. The vessel is left uncovered in a dark place, and the water changed daily. The sputum and eggs sink to the bottom of the vessel and are readily examined microscopically. Too much sputum is to be avoided as the development of the egg is hindered; test-tubes are unsuitable as the worms do not hatch well.

The egg of *Paragonimus westermanni* in fresh sputum is oblong (0.063 to 0.084 by 0.045 to 0.054 mm.) and yellowish brown (Fig. 1). It has an operculum at one end and contains an embryo and several yolk cells (Fig. 2). The yolk cells gradually enlarge and the granules manifest Brownian movement which lasts until the yolk cells are completely taken up by the embryo. At the ten cell stage

[1] These will be described fully below.

or a little later, the cell boundaries become indistinct. When the miracidium is about visible (Fig. 3), the yolk granules are diminished, and the contents of the egg become clearer. The rounded end of the ovoid embryo is directed toward the operculum and the oral part develops at that end (Fig. 4). At this stage the embryo resembles a melon seed and is covered with fine cilia. A few days later the embryo takes on a slow vermicular motion, becoming gradually more active until the miracidium bends upon itself. The cilia cover the surface of the body except the oral part, and are longest over the anterior part, especially about the protruding anterior end. They point posteriorly, except near the oral part where the direction is reversed. Through the vigorous movement of the cilia of the miracidium the operculum is torn off and the embryo is set free in the water (Fig. 5).

The egg containing the full grown miracidium measures 0.0792 to 0.09 by 0.0486 to 0.0567 mm., which is larger than that seen in the sputum. The mature miracidia measure 0.0612 to 0.072 by 0.036 to 0.045 mm.

The rate of development of the miracidia varies with the temperature and is retarded by cool weather. During the summer in Shinchiku, *i.e.*, from May to October, melon seed-like miracidia develop in 14 to 15 days, begin to move in 19 to 22 days, and hatch in 23 to 28 days. In March and April they take some weeks to hatch, and the miracidia remain for a long time within the egg even though they are as lively as in the warm season. From November to February or March no development was noted, though the eggs were watched constantly. According to my observations, the temperature for hatching is 25–31°C., and embryonic development ceases below 25°C. Manson (6) gives 26–34°C., Nakahama (7) 30°C., and Garrison and Leynes (8) 25–34°C. At 37°C. the eggs seem to disintegrate. The incubation period in summer is variable even in the same culture. Some eggs hatch in about 3 weeks, others as late as from 5 to 8 weeks. This difference may be due to other causes than temperature; possibly the length of time elapsing since the eggs were passed by the adult worm in the lungs of the patient before they were ejected with the sputum plays a part.

The miracidium when hatched measures 0.081 to 0.099 by 0.036 to 0.054 mm. It is provided with an alimentary tract and ganglion, two flame cells, and numerous granular embryonic cells. As it swims, the anterior end may not be pointed but invaginated. It can be kept alive for only a short time. In several hours its motion becomes sluggish and it assumes a ball-like appearance. Then it moves with a spiral motion and soon becomes inactive and dies.

Garrison states that direct sunlight is injurious to the miracidia. I also observed that embryonic development is arrested even in diffused light if this is intense. On the contrary, if the culture is kept in the dark, development goes on vigorously. Oxygen seems to be necessary for the development of the embryo. The vessel containing the eggs must be kept uncovered, and care should be taken

to change the water at least once a day. The eggs contained in sputum gradually become brown and die if dried, a space forming between the egg membrane and the shell. If the miracidia either within or without the egg membranes are subjected to 1 per cent hydrochloric acid, movement ceases and they die. It is known that they cannot resist the gastric juice.

In October, 1913, I introduced a quantity of water that contained full grown miracidia, either swimming free or still in the egg, into the stomach of pups; other pups were placed in the water containing free swimming miracidia. In neither case were the animals found to be infected by distomas, when killed and examined 45 to 100 days later. The miracidia appear to be unable to enter the animal body either *per os* or subcutaneously, unless they go through the second intermediate hosts.

The First Intermediate Host and the Cercaria of Paragonimus westermanni.

The cercariæ of *Paragonimus westermanni* were detected in the following three species of fresh water snails.

1. *Melania libertina* Gould (Fig. 20, a), which lives in pools or sluggish streams. In the villages of the savage mountain tribes, this species alone was found. Larger specimens than those seen in Shinchiku occur commonly in the main islands of Japan.

2. *Melania obliquegranosa* Smith (Fig. 20, b), which thrives in slowly flowing streams, is common in the flat region of Shinchiku.

3. *Melania tuberculata* Mueller, which occurs rarely in Shinchiku.

All the larger specimens of *Melania libertina* collected in the savage villages where pulmonary distomiasis occurs abundantly were found to be infected with the cercariæ. The occurrence of the cercariæ in *Melania obliquegranosa* collected in the lowlands parallels with the degree of infection. Cercariæ were found in only one specimen of *Melania tuberculata*, collected in Runasho village.

When the snails are placed in water containing miracidia, the latter swarm around them and become attached to the heads, jaws, and feet, and but rarely to the tentacles and mantles. They cling with their suckers, insert probosces into the tissues of the host and en-

ter the body of the snail like the cercariæ of *Schistosomum japonicum*, as described by Miyairi (9). Unlike the miracidia of *Schistosomum*, those of the pulmonary distoma shed their cilia in this act.

The cercaria measures 0.12 long by 0.09 mm. wide, and its tail is 0.054 mm. long. The oral sucker, 0.036 by 0.032 mm. in diameter, is provided with two pear-shaped bodies, the apices of which point posteriorly. It also has a spine, which appears to have a ring on its point. The posterior sucker is smaller than the oral one, having a diameter of 0.018 mm. The cercaria has three pairs of poison glands. The excretory vesicle of the glands is heart-shaped (Fig. 9).

Besides the cercariæ, sporocysts of various sizes are found abundantly in the liver of *Melania*. They are sometimes found in the heart and kidneys (Figs. 6 to 8, and 33).

The cercariæ mentioned have been detected in several specimens of *Melania* that thrive in the infected regions of the Prefectures of Niigata, Gifu, Tokushima, and Okayama on the main island of Japan.

The identification of the cercariæ with those of *Paragonimus westermanni* is based on the following data.

1. Only the cercariæ under consideration are found in the most thickly infected regions, among the savage tribes.

2. The miracidia of *Paragonimus westermanni*, which usually develop into the cercariæ described above, can enter *Melania* only.

3. The shape of the spine of the oral sucker of the encysted cercariæ in the crabs, and of the excretory vesicle of the young encysted cercariæ bears a striking resemblance to those of the cercariæ mentioned above.

4. The cercariæ successfully infected crabs free from distomas and developed into the specific encysted cercariæ.

The last fact seems to be the strongest evidence. The aquarium used for the experiment was a wooden box 3 feet long and 2 feet high. Three sides were completely covered by a thin cloth in order to prevent the intrusion of cercariæ from without. Of course, prior to the experiment it was ascertained that the cloth did not allow cercariæ to pass through. The cloth was covered by wire netting with meshes of $\frac{1}{3}$ mm., in order to protect it from being torn by the crabs. The bottom of the box was covered with sand and pebbles. The aquarium was placed in a stream so that the crabs might be kept in as natural a condition as possible. Some snails that had been shown to contain the cercariæ were kept with the distoma-free crabs for a time. Non-infected crabs were difficult to obtain. I failed

to secure distoma-free crabs from the streams that run through Formosa, even in those regions where pulmonary distomiasis does not occur. Finally I succeeded in collecting young *Potamon* (*Geothelphusa*) *obtusipes* and *Potamon* (*Geothelphusa*) *dehaanii*, and fifty specimens of the former and twenty of the latter were examined and found to be free from the encysted cercariæ. On September 4, 1915, the crabs and *Melania* were put together in the aquarium in the stream. Examinations were made daily. Most of the crabs climbed up the walls and were outside the water. Both the crabs and the snails began to die one after another. On October 10, 1915, I examined twenty specimens each of *Potamon obtusipes* and *Potamon dehaanii*. None of the former had encysted cercariæ, but one of the latter showed a few, some of which had not been in the host for many hours. 3 weeks later thirty-five specimens of *Potamon dehaanii* were examined, and three were found to be infected by young encysted cercariæ The experiments are being continued

Five young specimens of *Potamon obtusipes* and of *Potamon dehaanii* were placed in a vessel filled with water containing a large number of cercariæ, obtained by crushing snails. In 3 days one of the former species was found to harbor a few encysted cercariæ. It is evident that the infection occurred in the vessel; for the embedded, encysted cercariæ were so young that they could not have been many hours in the host. The reason why so few crabs become infected experimentally may be due to differences in environmental conditions.

Crabs as the Second Intermediate Host.

Seven species of fresh water crabs collected in the infected and non-infected regions were examined for encysted cercariæ. The following three species contained them.

1. *Potamon* (*Geothelphusa*) *obtusipes* Stimpson (Fig. 21), which is the first species in which the encysted cercariæ of *Paragonimus westermanni* were found, and which has only been found in the mountainous regions of Shinchiku. It seems probable that this species is peculiar to the regions mentioned above, though Stimpson points out that specimens collected in the island of Amamioshima in the Prefecture of Kagoshima are identical with them.

I found this species abundant in the streams running through the mountainous regions in the Prefecture of Shinchiku, and also present in the mountainous regions of Usekiko village in the Prefecture of Taichu and Chikutoki village in the Prefecture of Kagi. The occurrence in this species of encysted cercariæ corresponds with the degree of infection. Among the savages, where 30 to 50 per cent of the

population suffer from the infection, 80 to 100 per cent of this species harbored encysted cercariæ. Moreover, in Kalapai village, in which 55 per cent of the inhabitants suffer from pulmonary distomiasis, all the crabs harbored encysted cercariæ; while only 11 per cent of the crabs from the creeks of Naiwan, situated about 4 miles away, showed encysted cercariæ. The inhabitants of the latter village are believed to have the disease but rarely. In Sansaka, where distomiasis seldom occurs, the crabs showed no encysted cercariæ.

2. *Potamon (Geothelphusa) dehaanii* White (Fig. 22), which is found in the same locality, but less abundantly than the preceding species.

3. *Eriocheir japonicus* De Haan (Fig. 25) which, unlike the two species mentioned above, never occurs in the streams of the mountainous regions, but is found in the rivers flowing across the plain.

300 large specimens and over 30 small specimens were examined microscopically in February, 1915, for encysted cercariæ. Of these only two showed, in the gills, one encysted cercaria each. At first it was suspected that this was accidental, but later I learned that cercariæ may be found, though rarely, in the gills and muscles of this crab in the village of Torunsho.

Thus it seems to have been conclusively demonstrated that these three species of fresh water crabs act as the intermediate host of *Paragonimus westermanni;* the first species showed the largest percentage of infection, the second a much smaller percentage, while the third was but rarely infected. The first species is found exclusively in Formosa, while the second and third are found also in other parts of Japan. I came, therefore, to the conclusion that these last two species play the part of the second intermediate host in the main islands of Japan.

Recently, Kobayashi (10), Ando (11), and Yoshida (12) demonstrated that the second species is the intermediate host in the infected regions in the Prefectures of Niigata and Gifu, and the third species in the Prefecture of Tokushima, and both the second and third species in the Prefecture of Okayama. Yoshida (12) claims that another species of crab, *Sesarma dehaanii* Milne-Edwards (Fig. 24), is the second intermediate host in the village of Hiyeshima in Nishinaru County in the Prefecture of Osaka. This species is also found in the region near the sea in the Prefecture of Shinchiku in Formosa, but no encysted cercariæ are

found in it. Moriyasu, Arima, and Tanakamaru (13) lately detected a species of crab that harbors encysted cercariæ, of which they kindly sent me specimens. Upon examination, they were found to belong to the third species described above. Professor Miyairi of the Kyushu Imperial University also found that *Astacus japonicus* DeHaan is the intermediate host of *Paragonimus westermanni* in Korea. In one of the infected regions in Formosa, *i.e.*, in the village of Shinko, Prefecture of Taihoku, the third species as well as another variety, *Potamon* (*Parathelphusa*) *sinensis* Milne-Edwards (Fig. 23), was found by Yokokawa (14) to have encysted cercariæ. The following table shows the species and the localities in which they act as second intermediate hosts.

1. *Potamon* (*Geothelphusa*) *obtusipes* Stimpson.	Prefecture of Shinchiku.
2. *Potamon* (*Geothelphusa*) *dehaanii* White.	" " " Prefectures of Niigata, Gifu, and Okayama.
3. *Eriocheir japonicus* De Haan.	Prefectures of Shinchiku, Tokushima, and Okayama, and Korea.
4. *Sesarma dehaanii* Milne-Edwards.	Prefecture of Osaka.
5. *Astacus japonicus* De Haan.	Korea.
6. *Potamon* (*Parathelphusa*) *sinensis* Milne-Edwards.	Prefecture of Taihoku.

Encysted Cercariæ of Paragonimus westermanni in Crabs.

The size and shape of encysted cercariæ vary with their age. The younger ones are found embedded chiefly in the liver, while the older ones occur either in the liver, the gills, or even in the muscles. In the liver, they lie exclusively in the interspace of tissue.

1. Youngest Forms.—The youngest forms (Figs. 10 and 11) are not commonly detected in the crab, as the cercariæ usually enter at night. The youngest individuals present in the crabs were found at 5 o'clock in the morning just pushing their way into the parenchyma of the liver. They measured 0.13 by 0.05 mm. and had no tail. The posterior part is narrower than the anterior; they possess an oral sucker (0.04 mm. in diameter), and a spine in the oral sucker, which corresponds with that observed in the encysted cercariæ, except that it has no ring and a small posterior sucker (0.02 mm. in diameter) situated in the middle of the body. There is no excretory vesicle. In the anterior part of the body there is a tube-like organ which looks like the duct of poison glands. The worm appears as a white speck on the yellow parenchyma of the liver.

Immediately before transformation into encysted cercariæ the individuals are rarely seen, but not so rarely as the preceding stage. They now are folded on themselves and contract and extend their heads alternately but remain fixed

to the same spot. A little later a thin cyst, 0.11 mm. in diameter, encloses the worm, and within it the larva moves (Fig. 12). In still further advanced stages the cyst measures 0.13 mm. in diameter, the oral sucker is smaller, and a lumen (probably the pharynx) is seen immediately posterior to the latter. The posterior sucker has now enlarged slightly, while at the posterior extremity and along the median plane a rudimentary excretory vesicle, which sends off branches parallel to the axis of the body, has appeared (Fig. 13). Still later the cyst measures 0.14 mm. in diameter and there is a wider excretory vesicle, filled with coarse granules (Fig. 14). Finally, the larvæ lie straight within the cyst, the excretory vesicles gradually become heart-shaped, and fine dark granules appear and soon assume the form to be described.

2. Young Cercariæ (*Figs. 15 to 18*).—The young cercariæ possess a cyst measuring 0.18 to 0.2 mm. in diameter in which they lie extended. They have a large black excretory vesicle, and relatively large oral and posterior suckers, which tend to assume an ellipsoidal shape due to pressure. The oral sucker is 0.035 to 0.042 mm. long and 0.05 to 0.056 mm. wide, while the posterior sucker is 0.035 to 0.043 mm. long and 0.052 to 0.057 mm. wide. The spine of the oral sucker is present though it can be detected only with difficulty. No alimentary canal is differentiated. The cyst consists of chitin which is 0.005 to 0.006 mm. in thickness.

The young encysted cercariæ can be seen by the naked eye, in a piece of liver crushed between two slides, as small white dots lying between the lobules.

3. Full Grown Cercariæ (*Figs. 19 and 34*).—These are contained within a cyst from 0.26 to 1.0 mm. in length and 0.4 mm. in width. The oral sucker measures 0.07 to 0.09 mm. in diameter. The black-appearing intestinal canals are thick and wind on both sides of a large excretory vesicle. The posterior sucker (0.09 to 0.11 mm. in diameter) is a little larger than the oral one, and tends to be hidden by the excretory vesicle. The whole surface is covered with short cilia. The thick cyst, measuring 0.01 to 0.014 mm., is one of the characteristics of the species. The cercariæ move sluggishly within them. The liver usually contains three to four and never more than ten. The muscles contain few cercariæ; the gills contain the most. In the gills of one crab 97 full grown cysts were found. The cercariæ in this stage are detached from the gills and 20 per cent are found floating in the water. Under natural conditions they seem to leave the gills and drift along the streams, which thereby become a source of infection for human beings.

The above observations were made chiefly on *Potamon obtusipes*. In *P. dehaanii* and *Eriocheir japonicus* the development takes place in a similar way.

Experimental Infection with the Encysted Cercariæ.

In order to determine to what species the encysted cercariæ in the crab belong, tests were conducted on dogs. Pups born in the

regions in which pulmonary distomiasis does not occur were chosen, since dogs are liable to infection under natural conditions. Two pups were fed twice, on September 23, and October 10, 1914, with large quantities of the gills and livers of crabs containing encysted cercariæ. One of them died on December 9, 60 days after the last feeding.

Numerous cysts were found in the lungs. Each cyst showed two or three full grown distomas that contained no eggs in the uterus. They were 4 to 5 mm. long by 2 to 3 mm. wide. The other animal died on December 25, 75 days after the last feeding. Numerous cysts containing egg-bearing distomas were found in the lungs. The worms were 6 to 7 mm. long and 3 to 4 mm. wide, just one-half the size of the worms of spontaneous infection in the cat and dog and in man, but morphologically they were identical with *Paragonimus westermanni*.

The experiment was repeated on two pups from a non-infected region (December 26, 1914, to February 7, 1915) with identical results. On another occasion animals were given water containing the encysted cercariæ which had been detached from the gills of the crab, and adult distomas developed also in these animals.

The Course of Penetration of the Cercariæ in the Final Host.

Since the distomas appear to reach their destination in the final host by passing through the walls of the alimentary tract, the next step was to make clear their course. Microscopical examination of the viscera of the infected animals indicated that the distomas passed through the walls of the alimentary tract and the diaphragm, and thus reached the lungs. Yamagiwa (15) believes that the distomas reach the lungs by way of the liver, and then by the diaphragm, the pleura, etc., since adherent nodules involving these organs have been demonstrated. My observations were made in the early stages, so that congestion and hemorrhage of the mucous membrane of the intestine could be detected as well as petechiæ in the diaphragm and lungs, especially the lower lobes. By studying the petechiæ, points were found through which the worm seemed to have passed. At this early period I concurred with Yamagiwa's views. Just at

this juncture Yokokawa (14) discovered many distomas floating in the serous exudates of the abdominal and thoracic cavities of dogs to which encysted cercariæ had been fed.

At the suggestion of Dr. Miyajima of the Kitasato Institute, I examined a dog and a cat sent from Shinchiku where they had been fed encysted cercariæ, for more than 10 days, and succeeded in determining the path of penetration.

Experiment 1.—A pup was fed from April 15 to 27, 1915, large numbers of crabs gathered in the savage villages. The animal died on April 27. The body cavity was opened and the animal immersed in 10 per cent formalin.

Macroscopical examination showed petechiæ in the mucous membrane of the intestine near the jejunum. The other abdominal viscera and the diaphragm presented no changes. The lungs showed general pneumonic areas. Since this condition was not met with in other cases it must be regarded as a complication.

Nearly the whole omentum was sectioned, and several worms were found: one (0.22 by 0.14 mm., smaller than usual owing to the fixation) in the parenchyma of the omentum near the greater curvature of the stomach, another (0.36 by 0.24 mm.) in the fat tissue, and others in the diaphragmatic ligament where the worms (0.3 by 0.18 mm.) were surrounded by hemorrhage. No worms were found in the liver or the mediastinum, and none were detected in the lungs. Similarly, the contents of the lymphatic system, the thoracic cavity, and the heart were negative.

Experiment 2.—A cat was fed for 15 days with a large number of encysted cercariæ (April 18 to May 2, 1915), when it was chloroformed, the body cavity opened, and the animal placed in formalin.

On macroscopical examination petechiæ of various sizes, some as small as 1 mm. in diameter, were found in the mucous membrane of the jejunum (Fig. 26). A note by the collector of the material stated that four or five grayish white vesicles were present on the diaphragm, which undoubtedly were worms. Small petechiæ were present in the capsule of the spleen, and in the muscle of the diaphragm. The surface of the lungs, especially the lower lobes, showed many petechiæ.

Sections were made through the basal part of the omentum, the liver, the ligament and tendinous part of the diaphragm, and the mediastinal tissues, but no worms were found. Large portions of the lungs were sectioned and worms were found entering the pleura on their way to the parenchyma of the lungs. In sections made through the jejunum a worm (0.28 by 0.18 mm.) was found that had just entered the external layer of muscle. This path was indicated by the torn muscle fibers, the infiltrated round cells, and especially the eosinophil cells (Fig. 35). No worms were found in the cœlom; hence they must have dropped when the material was fixed. Worms could not be detected in the act of piercing

the lungs. However, in an experimental animal 7 weeks after the feeding, a worm (2.2 by 1 mm.) that had already reached the parenchyma of the lungs was found. Thus, it may be said that the path by which the worm reaches the lung parenchyma has been ascertained.

In brief, the results of my observations support Yamagiwa's view; for they establish the point that cercariæ of *Paragonimus westermanni* taken *per os*, with the food, pierce the intestinal wall, pass into the abdominal cavity, then through the diaphragm into the thoracic cavity, and finally reach the lungs, where they produce cysts and grow into adults.

Since my discovery, Yokokawa (16) has also reported in several papers, published in Japanese, minute observations on the same subject, and they have been further supported by Kobayashi (10) and Ando (11).

Additional experiments were carried out in Formosa, of which illustrative protocols are here given.

Experiment 3.—From April 18 to May 16, 1915 (29 days), a kitten[2] was fed with many encysted cercariæ and then killed for examination.

The mucous membrane of the intestines was found to be congested, and several petechiæ were seen in the jejunum. Several worms were attached to the omentum. The liver was hyperemic, and the upper portion in apposition with the diaphragm was perforated by small pores that reached the parenchyma, in which young distomas were seen wriggling in and out. Many younger ones were also attached in the same region. Several worms were attached to the liver and the ligament of the diaphragm, while some were seen crawling on the lower surface of the diaphragm itself (Fig. 28). The capsule of the spleen presented several cyst-like protuberances and some petechiæ. The kidneys were normal. By washing the abdominal cavity with saline solution, sixteen young distomas were obtained. From the liver and the diaphragm, eleven worms were obtained.

In the thoracic cavity about 30 cc. of bloody turbid fluid were present, which contained one worm. The parietal pleura showed many cysts and petechiæ. On the thoracic aspect of the diaphragm many punctures and hemorrhagic spots were seen and some worms were present in the muscle layer (Fig. 36).

Experiment 4.—From April 28 to May 16, 1915 (19 days), a kitten[2] was fed on crabs collected among the savages.

The surface of the liver and diaphragm showed numerous perforations in which were young wriggling worms. The muscular layer of the diaphragm was the

[2] These two animals were examined by my assistants in the Shinchiku Hospital during my stay in Tokyo.

seat of several hemorrhagic spots, as was also the visceral pleura. Thirty-six worms were found in the cœlom, and thirty-five on the surface of the liver.

Experiment 5.—100 mature encysted cercariæ were fed *per os* to a kitten at 10 a.m. and 4 p.m. on June 26, 1915. The animal was killed 6 hours later.

There were about 20 cc. of light yellowish fluid in the abdominal cavity; no worms were found. In the upper part of the ileum a young distoma that had just come out of the cyst, and another still in the cyst, were found; in the jejunum were found numerous specimens of *Ascaris mystax*. The other organs were normal. The fluke found in the ileum measured 0.35 by 0.21 mm. The empty cyst measured 0.28 mm.

Experiment 6.—A kitten was fed with 100 full grown encysted larvæ at 3 p.m. on June 27, 1915, and killed for examination at 2 p.m., July 17; that is, 3 weeks after the feeding.

The animal was somewhat emaciated. The abdominal cavity contained a small quantity of cloudy fluid; no worms were found. The serous membrane of the intestine was congested. One worm was attached to the surface of the liver. Three worms were seen moving on the abdominal surface of the diaphragm. A worm was found at the greater curvature of the stomach, but no worms were free within the abdominal cavity.

The thoracic cavity contained a large quantity of cloudy reddish fluid from which twenty worms were obtained. Two worms were attached to the heart. Many worms were found on the pleura where the sternum comes in contact with the pleural diaphragm. The lungs presented a light reddish color with a few scattered hemorrhagic spots. In the central portion of the lower lobe of the left lung one grayish cyst as large as the tip of the small finger was found. One or two worms were seen intruding into the pulmonary pleura, with here and there cyst-like protuberances.

The worms in the thoracic cavity and in the abdominal cavity are identical. The largest specimen measured 2.2 by 1.4 mm., and the smallest 1.1 by 0.8 mm.

Experiment 7.—A kitten was fed 200 full grown encysted larvæ at noon on July 1, 1915, and died at 6 p.m. on July 19, 19 days after the feeding. Most of the encysted larvæ fed were taken from the gills of somewhat decomposed crabs.

Emaciation; the abdominal cavity contained a small quantity of fluid. The intestinal serosa was slightly congested. One worm was attached to the upper parietal pleura; two worms were found on the liver, one attached to the serosa of the stomach, and another to the serosa of the duodenum. A very small worm was free within the abdominal cavity. The surface of the liver showed a number of vermicular scars. Two worms were penetrating the capsule of Glisson. One worm was attached to the esophagus. In the right lung were seen three somewhat large hemorrhagic spots. Within the pleural cavity opposite the sixth right intercostal space within a hyperemic area a worm lay wedged (Fig. 30).

No difference in size was noted between the worms in the thoracic and in the abdominal cavities. The largest specimen measured 1.5 by 0.7 mm., the smallest 1.0 by 0.6 mm.

Experiment 8.—A pup was fed with 227 full grown encysted larvæ at 8 p.m., July 8, 1915, and died at about 2 a.m., July 14, or 126 hours after the feeding.

A large quantity of light yellowish fluid containing two or three worms was found in the abdominal cavity. The intestines showed here and there ulcerations in the mucous membrane and innumerable *Ankylostoma caninum*. The stomach contained numerous ascarides. The liver showed two or three hemorrhagic spots, and similar areas occurred in the muscular layer of the diaphragm.

The thoracic cavity contained a large quantity of light yellowish fluid and a few worms. The lungs appeared pale, and a few hemorrhagic spots were seen. The worms in the abdominal and thoracic cavities were generally small, measuring 0.4 to 0.5 mm. by 0.2 to 0.3 mm.

Experiment 9.—A pup was fed fifty full grown encysted larvæ at 7 a.m., July 9, 1915, and died August 2; that is, 25 days after the feeding. The abdominal cavity was free from fluid. One distoma was attached to the upper left quadrant of the parietal peritoneum. The liver was hyperemic and showed a few serpentine scars on the surface. One worm was attached to the omentum. A few hemorrhagic spots were seen in the muscular layer of the diaphragm.

In the right thoracic cavity was a small quantity of bloody turbid fluid; there was none in the left. On the abdominal aspect of the diaphragm one active worm was seen, and another in the diaphragmatic ligament. The lungs showed numerous hemorrhagic spots but no cysts (Fig. 31).

The largest worm measured 3.0 by 1.2 mm., and the smallest 1.2 by 0.6 mm.

Experiment 10.—On September 9 and 10, 1915, a pup was fed 30 and 40 full grown encysted larvæ respectively, collected in a savage village. The animal was killed 45 days after the last feeding.

No fluid was present in the abdominal cavity. One worm was found on the abdominal surface of the diaphragm and another on the diaphragmatic surface of the liver. Both were small.

No fluid was present in the thoracic cavity. One worm was attached to the mediastinal tissues, and one to the pericardium. The lungs were reddish purple in color, and showed numerous dark brownish red hemorrhagic specks and gray cyst-like scars. The right lung contained about ten cysts, the left only one.

The largest worm measured 4.5 by 2.2 mm.; and the smallest was about one-half that size.

Experiment 11.—In order to determine whether the hairy crabs are the intermediate host of the worm, a pup was fed 200 crabs between July 27 and August 23, 1915, after the carapace and the legs had been removed, and the viscera and the muscles minced. The animal died at midnight on August 24. The crabs were bought in the market of Shinchiku and given to the animal without examination for the presence of the larvæ.

On the surface of the liver were one or two small hemorrhagic spots. In the right thoracic cavity was a small quantity of bloody fluid. One worm was attached to the lower lobe of the right lung, and one was seen crawling over the thoracic surface of the diaphragm. The lungs were pale with occasional large

dark bloody flecks, some of which had a wound in the central part where the parenchyma had been injured. In the inferior lobe of the right lung was seen a small dark red nodule, which had a small cavity in the center from 5 to 7 mm. in diameter and 12 or 13 mm. deep, at the bottom of which a worm was found.

In the ligament of the diaphragm were seen three small perforations the size of a pin-head. In the muscular layer of the diaphragm one or two hemorrhagic spots were present.

The worms in the thoracic cavity measured 3.0 by 1.5 mm., while those in the abdominal cavity measured 2.4 by 1.2 mm.

This experiment indicates that the hairy crabs may also act as the second intermediate host of *Paragonimus westermanni.*

Eighteen animals were employed[3] in the experiments, eleven of which are reported above.

The animals were given *per os* a comparatively small number of encysted larvæ, usually about 100. There developed in consequence mild infections with slight pathological changes.

Table I gives the results obtained.

TABLE I.

Results of Feeding Encysted Larvæ of Paragonimus westermanni to Kittens and Pups.

Experiment No.	Species.	Time between feeding and examination.	No. of worms.	Size of worms.
				mm.
1	Kitten.	6 hrs.	2	0.35 × 0.21
2	"	16 "		
3	"	1 day.	2	0.3 × 0.15
4	"	2 days.	1	0.73 × 0.21
5	"	2½ days.	1	0.5 × 0.22
6	Pup.	3 days, 15 hrs.	2	0.53 × 0.33 0.51 × 0.3
7	"	5 " 6 "	5–6	0.4–0.5 × 0.2–0.3
8	Kitten.	14 "	8	0.73–1.47 × 0.44–1.1
9	"	18 "	11	1.5–1.0 × 0.7–0.6
10	"	21 "	Over 25.	2.2–1.1 × 1.4–0.8
11	Pup.		4	3.0–1.2 × 1.2–0.6
	"	25 "	10	(No measurement taken.)
12	" *	45 "	4 (Besides numerous cysts.)	4.5 × 2.2

*Experiments in which the animals were kept longer than 45 days after the feeding are described elsewhere.

[3] With the exception of Experiments 1 to 4 and 11, cercariæ were given to the subject only once.

The following conclusions have been reached. Encysted larvæ taken *per os* by the final host reach the jejunum, where they emerge from the cysts and make their way through the intestinal epithelium into the submucosa (Figs. 26 and 35). From that structure they pierce the muscular layer and pass into the abdominal cavity. This process required, in the earliest case, 24 hours. Some larvæ remain in the intestines for 10 hours or more. Once in the abdominal cavity the worms proceed upward along the mesentery and the omentum and reach the liver (Figs. 27, 29, and 37), where they become attached to the diaphragm which is pierced at the ligament or in the muscular region (Figs. 28 and 36). The first worms pass into the thoracic cavity 77 hours after reaching the alimentary canal, but most of them remain on or in the liver for some time before they reach the thoracic cavity.

Some fail to reach the lungs, and, as Yokokawa believes, remain in the mesentery, the omentum, and the seminal ducts, forming cysts. In the thoracic cavity they often remain in the subvisceral pleural region or in the serous fluid. While in the pleural cavity they seem to grow before they enter the lung parenchyma where sooner or later they form cysts. It is doubtful whether they remain in the pleural cavity for a long time without forming cysts.

The changes in the lungs may be summarized as follows (Figs. 31, 32, and 38). 3 days after the feeding, a few pin-head hemorrhagic spots appear in the lungs. They are the beginning of wounds caused by the worms. In about 14 days, some of these spots become dark red, and in 21 to 25 days pale cysts have developed. Usually the cysts correspond in position to the petechiæ. The following phenomena have also been observed. In some instances, 45 days after feeding the encysted cercariæ, on the surface of the lungs numerous small dark red cysts are formed about which are infiltrations of polynuclear leukocytes or round cells in addition to the red corpuscles. In 50 days the surface of the lungs comes to have numerous dark reddish hard cysts as large as the tip of the little finger. The cut surface of the cyst appears dark red, from a previous hemorrhage; in the center is a vacuole the size of a pea. The distomas when present lie in the vacuole; but empty ones are also observed, for the worms may escape into the neighboring tissues. After about 90

days, the cysts have become bluish gray. On section a vacuole as large as a bean is found, in which porridge-like matter or one or two mature distomas are seen. In the latter case one of the two may be found dead. The walls of the cyst are composed of a thin layer of connective tissue.

The cyst wall may in some regions be wanting, the lumen being connected directly with the air spaces, a bronchus, or radicle of the pulmonary vein. Thus it is seen how the regional bleeding or pneumonic infiltration of the alveoli originates. Eggs were observed in worms 90 days after the feeding. When they are shed, they lie sometimes in the cysts, but oftener in the parenchyma of the lungs, mingling with the erythrocytes and leukocytes, epithelial cells of the lungs, or cellular detritus, etc. The bronchi and bronchioles near the lesions are dilated and contain erythrocytes or leukocytes together with eggs. The microscopical changes described indicate that the worms do not always remain within the cysts, but may emigrate into other parts of the lungs.

Development of Cercariæ in the Final Host.

The size of the distoma depends upon (*a*) the initial size of the cercariæ and (*b*) the nutriment. The following figures give the measurement of specimens fixed with alcohol.

TABLE II.

Size of Paragonimus westermanni at Various Ages.

Age.	Length.	Breadth.
days	*mm.*	*mm.*
Just hatched.	0.3 –0.48	0.18–0.23
3	0.44–0.48	0.23–0.26
6	0.44–0.53	0.26–0.32
14	0.65–0.95	0.37–0.61
18	0.8 –1.6	0.4 –0.85
21	1.3 –2.0	0.8 –1.1
25	0.9 –2.2	0.7 –1.3
45	2.5 –3.5	1.5 –2.0
60	4.0 –5.0	2.0 –3.0
90	6.0 –7.0	3.0 –4.0

Young worms (Figs. 39 to 42) have relatively large suckers, as shown by the measurement of fresh specimens. The larvæ which have just left the cysts present an oral sucker whose diameter is 0.07 to 0.09 mm. and a ventral sucker 0.09 to 0.11 mm. in diameter. In a 14 day old specimen (*i.e.*, after the feeding) the oral sucker is 0.14 to 0.23 mm. and the ventral sucker 0.16 to 0.26 mm.; in 21 day old specimens the oral sucker is 0.22 to 0.33 mm. and the ventral sucker 0.23 to 0.36 mm.; in those 45 days old the oral sucker is 0.3 to 0.5 mm. and the ventral 0.33 to 0.53 mm. in diameter.

The encysted cercariæ have a large excretory vesicle, occupying nearly the whole central body space, which is filled with a black granular substance. In the final host the contents escape and the vesicle becomes a small dark-appearing space.

The development of the genital organs takes place slowly. In a young worm just out of the cyst, the gonads are hardly visible. In a 2 week specimen gonidial regions appear which are deeply stained by borax carmine. In 18 to 21 day specimens the differentiation of the ovaries and the uterus has taken place. The ootypes appear as small groups of cells, one on each side of and posterior to the ventral sucker. The uterus is a simple winding tube. The testes make their appearance at a region corresponding to the ovaries. In a 25 day specimen (Fig. 42) both the ovaries and the testes have sent out several branches. In 50 day specimens (Fig. 43) the gonads are mature, but the yolk glands are not yet fully developed. The 90 day specimens (Fig. 44) lay eggs and have mature yolk glands. To sum up, within 3 weeks the rudimentary genital organs make their appearance and within 3 months they begin to function.

Resistance of the Encysted Cercariæ to Environmental Influences.

It is of importance for the prevention of pulmonary distomiasis to determine the power of resistance of encysted cercariæ to external influences.

Young encysted cercariæ are so delicate that they die in a few hours after becoming detached from a crab. They never develop in the final host, a fact experimentally proved by feeding them to a dog.

If full grown encysted cercariæ are artificially removed from the gills of the crabs and put into clear water, they soon swell and curl within the cysts similarly to other distomas. If the cercariæ are small, they remain inactive for a day or two or even longer within the cysts. Encysted cercariæ kept in clear water for almost 3 days either remain curled up or develop a remarkably enlarged excretory vesicle and compressed intestine. None wriggled, but one or two dead worms were seen to have shed the cysts. On another occasion

the encysted cercariæ still attached to the gills were put into the water and allowed to stand for some time at room temperature (about 30°C.). After 3 days the results were as shown in Table III.

TABLE III.

Behavior of Encysted Cercariæ in Water.

No. of gills.	Total No. of encysted cercariæ.	Empty cysts.	Dead cysts.	Living cysts.	Free cercariæ.
1	16	7	3	6	1
2	6	3	1	2	—
3	7	2	1	4	1
4	3	1		2	1
5	3	1	1	1	—
6	7	5	—	2	1
Total.	42	19 (45.2%)	6 (14.3%)	17 (40.5%)	4 (9.5%)

From this table it is seen that about half the encysted cercariæ hatched, while the other half remained within the cysts although still alive. A few were found dead inside the cysts. The remainder of the cysts, reexamined 5 days later, were found either evacuated or dead.

As Kobayashi (5) has shown with *Clonorchis sinensis*, the encysted cercariæ of *Paragonimus westermanni* leave the cysts on being kept several hours in fresh water, and the empty cysts are often seen in the alimentary canal of the final hosts, and it may therefore be inferred that the cercariæ escape before the cysts are digested in the intestine. In the winter, however, when the temperature in Shinchiku is about 15°C., the cercariæ do not escape from the cysts even if kept in fresh water for 2 weeks or more.

The question arises whether the cercariæ would infect herbivorous animals since they are liberated without any external agency. Ando (17) reported the successful infection of white mice, guinea pigs, and rabbits, to which encysted cercariæ had been fed. I also made experiments upon mice, but have reached the conclusion that the infection seems more difficult to achieve in these small animals than in dogs and cats.

The newly hatched cercariæ do not resist even slight temperature changes.[4] In the summer in Shinchiku when the room temperature is about 30°C. they all die in 20 to 30 hours. As long as the encysted cercariæ remain in cold streams ready to enter the final host, they do not become liberated. It is of interest from the standpoint of the prevention of distomiasis that the free, living cercariæ may be swallowed by man with water or food without harm.

On the other hand, the full grown cercariæ are enclosed within cysts much thicker than those of any other known species. The cysts are impermeable to both paraffin and celloidin. The resistance was tested with results which may be stated as follows:

When the crabs containing them are roasted over the fire until the muscles turn white the cercariæ are killed. Heating the crabs in water at 55°C. for 5 minutes also destroys them.

Heating the encysted cercariæ, removed from the crab, in water at 45°C. for 37 minutes does not kill them, but after heating to 55°C. for 10 minutes or to 70°C. for 5 minutes, they are killed.

The encysted cercariæ survive in 1 per cent solution of sodium chloride for 3 hours, or in 10 per cent solution for 2 hours. They first shrink but are soon restored to normal when transferred to fresh water.

The encysted cercariæ contained on the crabs' gills were put in soya sauce. At the end of 30 minutes they were alive, but after 9 hours they were killed. 50 specimens of the larger encysted cercariæ which had been immersed in soya sauce for 2 hours were fed to a kitten which when killed 3 weeks later was found not to be infected.

Immersion in vinegar for 30 minutes does not kill the cercariæ, but immersion for an hour does.

The above experiments may be summarized as follows: The cercariæ are liberated under natural conditions, if the temperature is high enough. When hatched they have little power of resistance to injurious influences. Within the cysts, on the contrary, they are hardy and withstand for quite a time immersion in solutions of table salt, soya sauce, and vinegar. Hence, crabs are dangerous unless they have been in soya sauce or vinegar for 2 hours or more after their carapaces are removed. A solution of table salt at the con-

[4] This was confirmed by the experiments of Matsui, Y., Biological studies on the cercariæ of the pulmonary distoma, *Hokuyetsu Igakkwai Zasshi*, 1915, xxx, No. 3.

centration used for culinary purposes is not strong enough to kill cercariæ. Though the cercariæ are not especially resistant to heat, half boiled or half roasted crabs are unsafe as food.

Principal Causes of the Prevalence of Pulmonary Distomiasis and Its Prevention.

It still remains to be determined how human pulmonary distomiasis is caused. At first I entertained the notion, in conformity with that of Ando (11) and Moriyasu, Arima, and Tanakamaru (13), that the encysted cercariæ, separated from the gills, which survive for a time on the surface of the water and which may be found free in water in which infected crabs have been, are the sources of infection. But later I concluded that the eating of crabs containing the cercariæ is the chief cause of the disease. Of twenty-two patients suffering from the disease, seventeen, or 77 per cent, gave a history of eating hairy crabs and three, or 13.6 per cent, red crabs.

An objection to this view may be found in the fact that only about 0.5 per cent of the crabs are infected. But a similar condition is found in liver distomiasis, for Kobayashi (5) has affirmed that the least infected fish, *Carassius auratus*, is responsible for most of the hepatic distomiasis. This view is supported by the statement that raw crabs are eaten in some parts of Japan and particularly in the highly infected regions of Korea.

Hence it may be concluded that the eating of raw or imperfectly prepared crabs and the drinking of river water which they inhabit are the two principal causes of pulmonary distomiasis.

CONCLUSIONS.

1. The morbidity of pulmonary distomiasis among the school children in the plains of the Prefecture of Shinchiku is 4.3 per cent, while in the mountainous regions among the savages it reaches in some districts 50 per cent.

2. Seventeen species of cercariæ were discovered in fresh water mollusks in the Prefecture of Shinchiku, Formosa. But it was impossible to ascertain from morphological characteristics alone which of them developed into the pulmonary fluke. Consequently,

the eggs of the pulmonary fluke after hatching into miracidia were allowed to come into contact with several species of fresh water mollusks, of which they infected two. But as it was difficult to keep the two species alive in the aquarium long enough to get cercariæ, the second intermediate hosts of the pulmonary distomas were looked for in the severely infected villages of the savage tribes.

3. The miracidia of the pulmonary distomas leave the egg about 4 weeks after they are first set free in the water, and if they do not reach mollusks they soon die.

4. Three species of fresh water mollusks were found to act as the first intermediate host of the pulmonary distomas; *viz.*, *Melania libertina* Gould, *Melania tuberculata* Mueller, and *Melania obliquegranosa* Smith.

5. The cercariæ of the pulmonary distoma may be identified by their small size and a spine in the oral sucker. They develop in the liver of the three species of *Melania* mentioned above.

6. The second intermediate hosts of the pulmonary distoma, detected in the Prefecture of Shinchiku, are the following three species of fresh water crabs: *Potamon* (*Geothelphusa*) *obtusipes* Stimpson (native name, red crab), *Potamon* (*Geothelphusa*) *dehaanii* White (native name, dung crab), and *Eriocheir japonicus* De Haan (native name, hairy crab). In addition it was discovered that the following two species might act as intermediate hosts: *Sesarma dehaanii* Milne-Edwards and *Potamon* (*Parathelphusa*) *sinensis* Milne-Edwards. In Formosa four of the five species are the carriers of the cercariæ.

7. The encysted cercariæ are found in the gills, liver, and muscle, and have an elongated dark excretory vesicle in the middle of their bodies. They resemble the adult flukes.

8. Full grown encysted cercariæ fed to dogs develop into mature pulmonary distomas and begin to lay eggs in about 90 days.

9. In the final host the parasites are taken into the alimentary canal as encysted cercariæ. They liberate themselves from the cysts in the intestine and bore through the jejunum into the abdominal cavity. They then pierce the diaphragm, enter the thoracic cavity, and piercing the pleura reach the lungs. In the parenchyma of the lungs they form cysts and develop into adult forms.

10. The chief causes of pulmonary distomiasis are the eating of raw or insufficiently cooked crabs infected with the cercariæ of *Paragonimus westermanni*, and the drinking of river water containing them.

In conclusion the author wishes to express his indebtedness to Professor Takaki, Chief of the Scientific Research Institute of the Taiwan Government, and others who have given him valuable assistance.

BIBLIOGRAPHY.

1. Ringer, cited in Inouye, Z., Ueber das Distomum Ringeri (Cobbold), *Z. klin. Med.*, 1903, l, 120.
2. Nagano, J., Ten years' experience in Formosa, Formosa, 1910.
3. Nakagawa, K., On pulmonary distomiasis that prevails in the Prefecture of Shinchiku, *Taiwan Igakkwai Zasshi*, 1915, No. 138; Abridged report on the discovery of the intermediate host of the pulmonary distoma, *Tokyo, Iji Shinshi*, 1915, No. 1910; On the study of the development of the pulmonary distoma, *Chyugwai Iji Shimpo*, 1915, Nos. 843, 845; The mode of infection in pulmonary distomiasis, *J. Infect. Dis.*, 1916, xviii, 131.
4. Matsuo, M., and Yokokawa, S., Results of the statistical observations on the visceral parasites among the natives of Formosa, *Taiwan Igakkwai Zasshi*, 1912, No. 114.
5. Kobayashi, Harujiro, On the study of the liver distoma, *Saikin Gaku Zasshi*, 1912, No. 202.
6. Manson, P., Distomum ringeri, *Med. Times and Gaz.*, 1881, ii, 8; 1882, ii, 42.
7. Nakahama, cited in Inouye, Z., Ueber das Distomum Ringeri (Cobbold), *Z. klin. Med.*, 1903, l, 120.
8. Garrison, P. E., and Leynes, R., The development of the miracidium of *Paragonimus* under various physical conditions, *Philippine J. Sc., B*, 1909, iv, 177.
9. Miyairi, K., cited in Ando, R., A new endemic region of pulmonary distomiasis in the Prefecture of Gifu and the intermediate host of the parasites and encysted cercariæ of the same, *Chyugwai Iji Shimpo*, 1915, No. 845.
10. Kobayashi, Hisao, On the second intermediate host of the pulmonary distoma and the encysted cercariæ (a preliminary report), *Tokyo Iji Shinshi*, 1915, No. 1918; On pulmonary distomiasis occurring in the Prefecture of Niigata, *Tokyo Iji Shinshi*, 1915, Nos. 1925, 1930.
11. Ando, R., A new endemic region of pulmonary distomiasis in the Prefecture of Gifu and the intermediate host of the parasites and encysted cercariæ of the same, *Chyugwai Iji Shimpo*, 1915, No. 845.

12. Yoshida, S., On the intermediate host of the pulmonary distoma that occurs in the Prefectures of Osaka and Tokushima, *Osaka Igakkwai Zasshi*, 1915, xix, No. 9; On the intermediate host of the pulmonary distoma that occurs in the Prefecture of Okayama, *ibid.*, 1915, xix, No. 10.
13. Moriyasu, R., Arima, Y., and Tanakamaru, J., Study on pulmonary distomiasis in Korea. The first preliminary report, *Tokyo Iji Shinshi*, 1915, No. 1941.
14. Yokokawa, S., On the penetration path of the pulmonary distoma in experimental animals, *Tokyo Iji Shinshi*, 1915, No. 1920.
15. Yamagiwa, K., Lectures on general pathology, Tokyo, 1909, ii.
16. Yokokawa, On the penetration path of the pulmonary distoma in the final host, *Taiwan Igakkwai Zasshi*, 1915, Nos. 152, 153.
17. Ando, Study on pulmonary distomiasis occurring in the Prefecture of Niigata, *Tokyo Iji Shimpo*, 1915, Nos. 847, 851.

EXPLANATION OF PLATES.

PLATE 22.

Early developmental stages of *Paragonimus westermanni*.

FIG. 1. Eggs in fresh sputum. Zeiss oc. 2, obj. $\frac{1}{12}$, oil immersion.

FIGS. 2 to 4. Eggs in various stages of development. Zeiss oc. 2, obj. $\frac{1}{12}$, oil immersion.

FIG. 5. Free swimming miracidia. Zeiss oc. 2, obj. $\frac{1}{12}$, oil immersion.

FIGS. 6 to 8. Sporocysts in *Melania libertina*. Zeiss oc. 2, obj. $\frac{1}{12}$, oil immersion.

FIG. 9. Cercaria developed in *Melania*. Zeiss oc. 2, obj. $\frac{1}{12}$, oil immersion.

FIGS. 10 to 14. The youngest cercariæ that lie in the liver of the fresh water crab, *Potamon obtusipes*. Zeiss oc. 2, obj. AA.

FIGS. 15 to 18. Encysted young cercariæ in the liver of a crab. Zeiss oc. 2, obj. AA.

FIG. 19. Full grown encysted cercaria. Zeiss oc. 2, obj. AA.

PLATE 23.

FIG. 20. Fresh water univalves, the first intermediate host of *Paragonimus westermanni*. Natural size. *a*, *Melania libertina* Gould; *b*, *Melania obliquegranosa* Smith.

FIG. 21. Fresh water crab, *Potamon obtusipes* Stimpson, the second intermediate host of *Paragonimus westermanni*. Natural size.

FIG. 22. Fresh water crab, *Potamon dehaanii* White, the second intermediate host of *Paragonimus westermanni*. Natural size.

PLATE 24.

The second intermediate hosts of *Paragonimus westermanni*.

FIG. 23. *Potamon sinensis* Milne-Edwards. Natural size.

FIG. 24. *Sesarma dehaanii* Milne-Edwards. Natural size.

FIG. 25. *Eriocheir japonicus* De Haan. Natural size.

PLATE 25.

FIG. 26. Jejunum of a kitten experimentally infected by *Paragonimus westermanni*. Natural size. *a*, petechiæ; *b*, mesentery.

FIG. 27. Omentum of the same film to show the worm attached on the surface. Natural size. *a*, young worm; *b*, stomach.

PLATE 26.

FIG. 28. Diaphragm of a kitten that had been experimentally infected by *Paragonimus westermanni*. Natural size. *a*, young worm just piercing through the muscular layer; *b*, petechiæ produced by the worm; *c*, tendinous region of the diaphragm.

FIG. 29. Liver of the animal shown in Fig. 28. Natural size. The brown irregular lines indicate the path of the young worm.

PLATE 27.

FIG. 30. Intercostal muscle of a kitten that had been experimentally infected by *Paragonimus westermanni*. Natural size. *a*, ribs; *b*, intercostal muscle; *c*, young worm.

FIG. 31. Lungs of a pup experimentally infected by *Paragonimus westermanni*. The specimen was examined a few days after feeding. Natural size. *a*, petechiæ.

FIG. 32. Lungs of a pup experimentally infected by *Paragonimus westermanni*, 50 days after feeding. Several cysts are present. Natural size. *a*, cysts.

PLATE 28.

FIG. 33. Section of the liver of *Melania libertina* that harbors the cercariæ. *a*, liver parenchyma; *b*, cercariæ. Zeiss oc. 2, obj. DD.

FIG. 34. Section of the gills of *Potamon obtusipes*, infected by encysted cercariæ. *a*, encysted cercariæ. Zeiss oc. 2, obj. AA.

PLATE 29.

FIG. 35. Section of the jejunum of a kitten experimentally infected. *a*, mucous membrane; *b*, connective tissue; *c*, internal muscular layer; *d*, external muscular layer; *e*, young distoma piercing through the intestinal wall; *f*, worm track. Zeiss oc. 2, obj. AA.

FIG. 36. Section of the diaphragm of a kitten experimentally infected. *a*, young worm in the muscle of the diaphragm. Zeiss oc. 2, obj. AA.

PLATE 30.

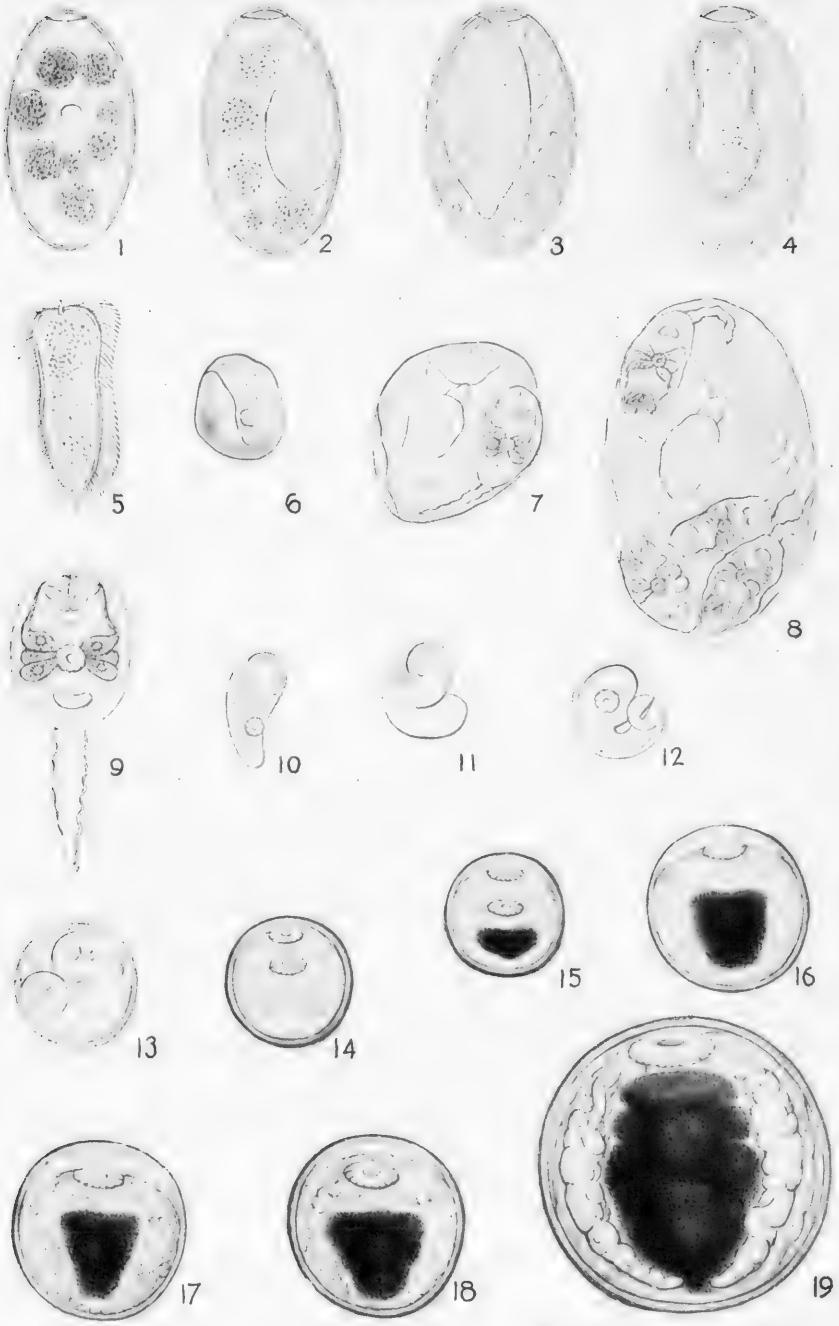
FIG. 37. Section of the liver of a kitten experimentally infected, showing the hemorrhagic spots caused by the young worm. *a*, hemorrhagic spots. Zeiss oc. 2, obj. AA.

FIG. 38. Section of the lung of a pup experimentally infected; 60 days after feeding. *a*, half grown worm. Zeiss oc. 2, obj. AA.

PLATE 31.

Various developmental stages of *Paragonimus westermanni* in the final host.

FIG. 39. Young worm, 3 days after feeding. × 30.
FIG. 40. Young worm, 14 days after feeding. × 30.
FIG. 41. Young worm, 21 days after feeding. × 30.
FIG. 42. Young worm, 25 days after feeding. × 30.
FIG. 43. Half grown worm, 50 days after feeding. × 15.
FIG. 44. Mature worm 90 days after feeding. × 15.



(Nakagawa: Pulmonary distomiasis.)

20

21

22

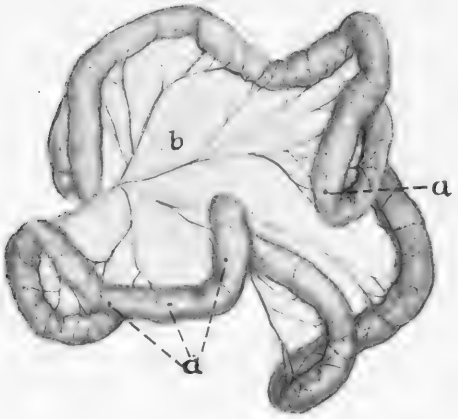
(Nakagawa: Pulmonary distomiasis.)

23

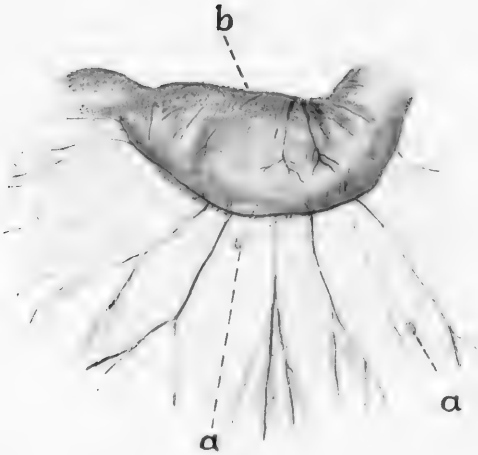
24

25

(Nakagawa: Pulmonary distomiasis.

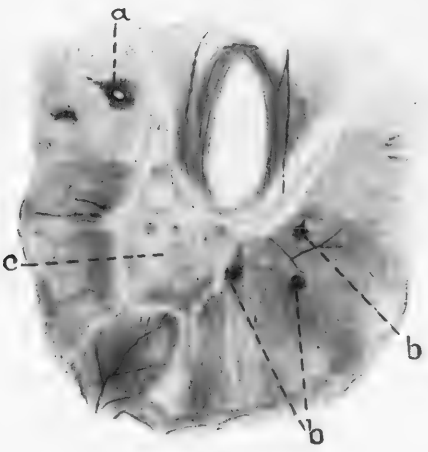


26

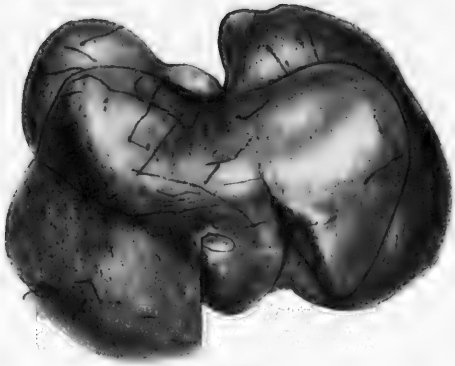


27

(Nakagawa: Pulmonary distomiasis.)

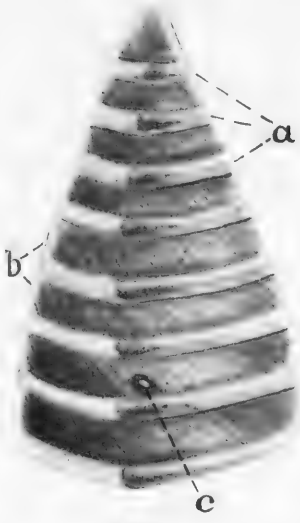


28

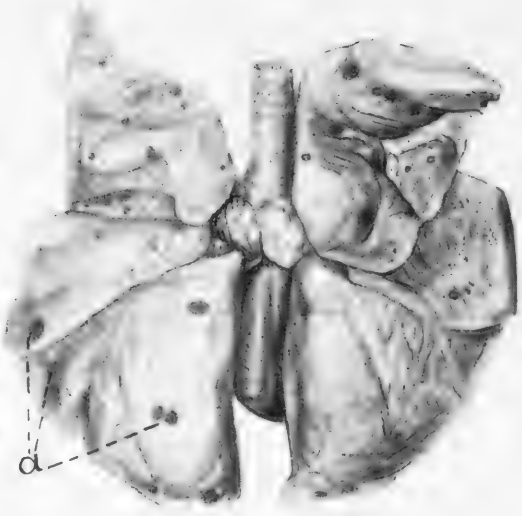


29

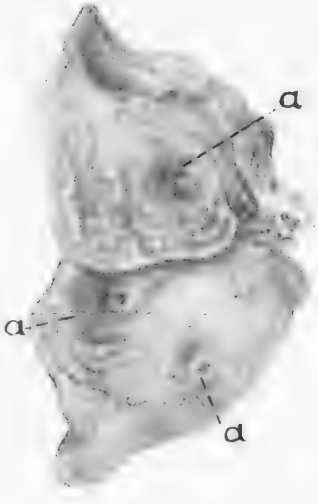
(Nakagawa: Pulmonary distomiasis.



30



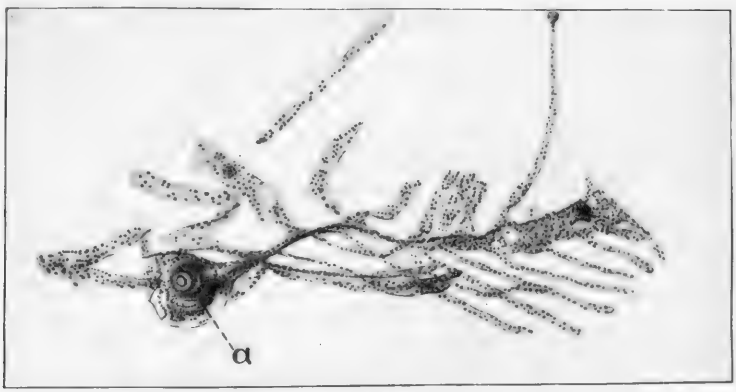
31



32

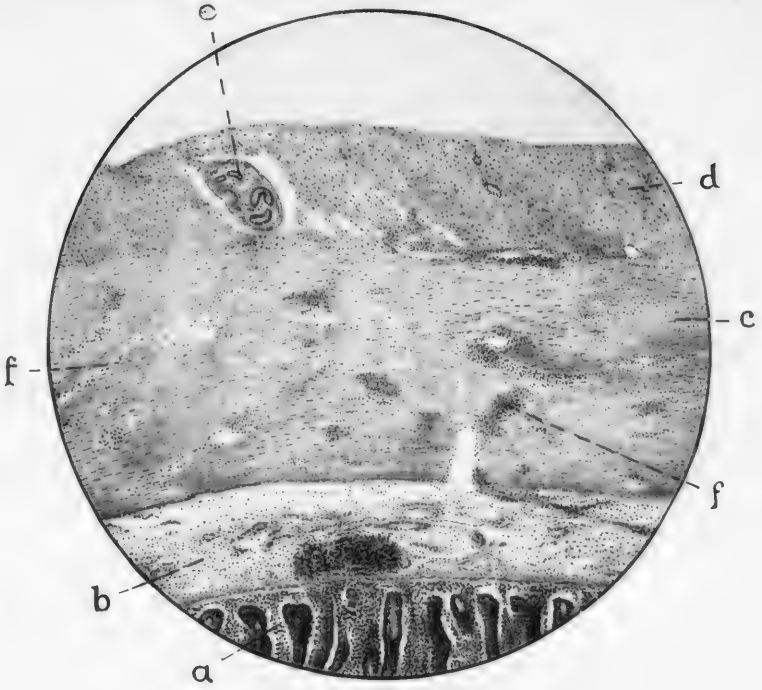
(Nakagawa: Pulmonary distomiasis.)

33

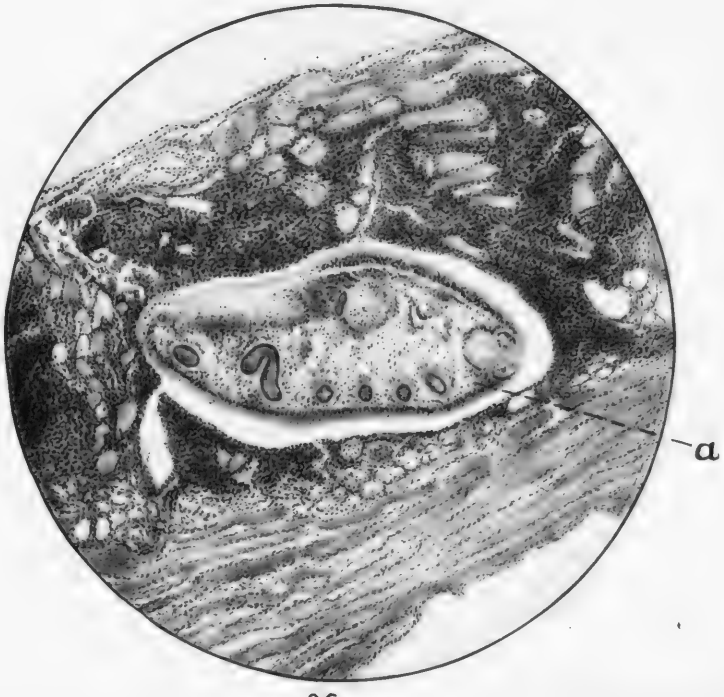


34

(Nakagawa: Pulmonary distomiasis.)

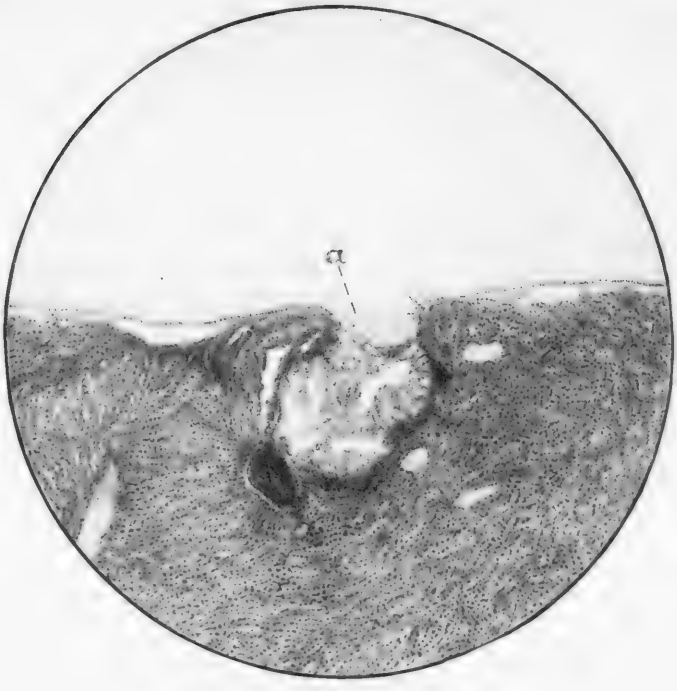


35



36

(Nakagawa: Pulmonary distomiasis.)



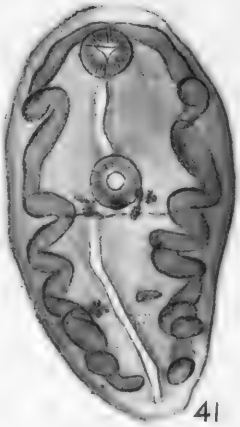
37

38

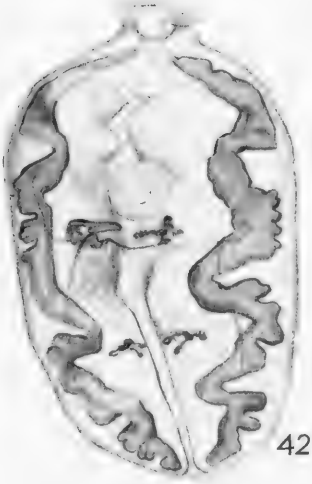
(Nakagawa.: Pulmonary distomiasis.)

39

40



41



42

43

44

(Nakagawa: Pulmonary distomiasis.)

THE DISTRIBUTION IN THE HUMAN BODY OF SPIROCHÆTA ICTEROHÆMORRHAGIÆ.*

By RENJIRO KANEKO, M.D., and KIKUZO OKUDA, M.D.

(*From the First Medical Clinic of the Imperial University in Kyushu, Fukuoka, Japan.*)

(Received for publication, February 20, 1917.)

The material examined came from autopsies performed on forty-three patients who died at various stages of illness, as shown in the table. The ages ranged from 2 to 73 years. There were 36 males and 7 females. 5 patients had cirrhosis of the liver, 9 a mixed infection. In 19 cases, serum was administered in varying quantities; 1 was treated with salvarsan. In a number of cases, only the vital organs were examined. As a rule, these organs were placed in a 10 per cent formalin solution; a few were treated with Orth's fluid. All the material examined was impregnated with silver, according to Levaditi's method.

General Distribution of the Spirochetes.

Inada[1] and Ido have divided the progress of Weil's disease into three clinical stages; (1) febrile, (2) icteric, (3) convalescent. As the distribution of the spirochete varies in the different stages, which are not sharply demarcated, we shall discuss the findings for each stage separately.

Febrile Stage.—This period continues to the 6th or 7th day of illness. The clinical symptoms are fever, intestinal disturbances, headache, muscular pains, hyperemia of the conjunctiva, and albuminuria. The blood is pathogenic for guinea pigs. No immune bodies could be demonstrated by Pfeiffer's test.

Spirochetes in large numbers are present. Their distribution resembles in general that in the experimental guinea pigs. The organisms are lodged mainly

* Presented in April, 1916, before the Japanese Congress on Internal Medicine and the Japanese Pathological Congress.

[1] Inada, R., The clinical aspects of spirochætosis icterohæmorrhagica or Weil's disease, *J. Exp. Med.*, 1917, xxvi, 355.

in the liver, kidneys, and suprarenals. Immune bodies are present as early as the 5th day, and we may assume that they exist even prior to that time, but as death does not usually occur so early in the disease, opportunity was lacking for substantiating this point. In the patient treated with salvarsan, who died on the 6th day,[2] spirochetes were found in the liver. That organ showed cirrhotic changes, and it is possible that the irregular distribution is related to this condition, although a similar distribution was found in a patient dying on the 8th day, as well as in the animals treated with serum or salvarsan. We believe that the destruction of spirochetes in the liver and their irregular distribution are due to the presence of immune bodies. The spirochetes in the suprarenals of this patient were distributed in groups.

By the 7th day the spirochetes in the liver have been almost completely destroyed, and are found but rarely thereafter.[3] We found only a few in 2 cases on the 7th day, in 5 cases on the 8th day, and in 8 cases on the 9th day of illness.

In the suprarenals spirochetes have not been clearly demonstrated on the 7th day, but it is certain that by the 8th day their almost complete destruction has been attained. The organisms are usually found in the kidneys on the 6th and 7th days.

Numerous spirochetes were also found in the pancreas, cardiac muscles, in the intestinal wall, the prostate, testicles, epididymis, the walls of the urinary bladder, and the arteries. The spleen and the lymph glands showed, also in the experimental animals, a small proportion of spirochetes.

The distribution of *Spirochæta icterohæmorrhagiæ* in human beings on the whole parallels that found in guinea pigs, with the exception that in man the organisms are more loosely and irregularly scattered and also show a greater degree of degeneration. These differences are attributable to the immune bodies. It is evident from the infection experiments made with the blood of patients that development of immune bodies is already under way as early as the 5th or the 6th day. No opportunity has presented itself to investigate the distribution of spirochetes at the beginning of illness, before the immune bodies have appeared, but recently we observed a patient who on the 4th day showed numerous spirochetes in the blood, similar to the findings in the guinea pig.

[2] This case was kindly put at our disposal by Dr. Takeya, Chief of the Second Medical Clinic, to whom we are greatly indebted.

[3] Dr. Iida, Assistant in the Second Medical Clinic, in one case found an abundance of spirochetes in the liver of a patient dying on the 8th day.

Icteric Stage.—This stage covers a period from the 7th or 8th to the 12th or 13th day of illness. The icteric condition is then at its height and the mortality is greatest. The proportion of autopsies was twenty-one out of forty-three cases. Further development of the immune bodies takes place during this stage, and they can be demonstrated in the blood.

In the course of this period the spirochetes disappear from the blood, and infection experiments are usually negative. Destruction of the spirochetes in the organs takes place. They disappear almost completely from the liver and the suprarenals, leaving only a slight degenerated residue,[4] but can be traced more or less readily in the kidneys, cardiac muscles, skeletal muscles, particularly the gastrocnemius, and rectus abdominis, the walls of the intestine, especially the appendix, large intestine, and stomach, prostate, urinary bladder, testicles, and epididymis, thymus, and uterine muscles. They are most constantly found in the kidneys and the cardiac muscles, but even here the organisms are not very numerous.

On the whole, the spirochetes during this stage are more abundant in the cells (epithelial, muscular, etc.). Those located in the kidneys are found in the tubules. There are massed foci in the interstitial spaces. Frequently the spirochetes are found in coagulated, homogeneous, or hyaline substance, such as urinary casts, degenerated hyaline muscle cells, etc.

Convalescent Stage.—This stage begins variously from the 13th to the 16th day of illness. The immune bodies are then fully developed, and spirochetes are abundantly excreted with the urine.

---

[4] The abundance of spirochetes in Dr. Iida's case (8th day) must be considered exceptional.

In the table the following signs are used:

* Cirrhosis of liver.

† Cirrhosis and cancer of liver.

‡ Mixed infection.

+, very sparse distribution of spirochetes; 1 or a few to a specimen. **+**, sparse distribution. Spirochetes can be found readily; 10 to 20 to a preparation, 1 in one or more fields. **+ +**, spirochetes relatively numerous, 1 or more to a field, many in a preparation. **+ + +**, spirochetes numerous; some in every field; perhaps many in a single field. −, negative. No spirochetes in 1 or more specimens.

The immune serum was given chiefly intravenously; the figures in bold-faced type indicate subcutaneous administration.

Case 12 was placed at our disposal through the kindness of Dr. Iida, Assistant in the Second Medical Clinic of the University; Case 41 by Mr. Onuma, Director of the Chibaken Board of Health. We desire herewith to express our thanks.

No.	Age.	Sex.	Day of illness.	Hours until autopsy.	Treatment.	Fixing fluid.	Liver. Extracellular.	Liver. Intracellular.	Gall bladder and ducts.	Kidney. Interstitiae.	Kidney. Tubules.	Suprarenals.	Spleen.	Lymph glands.	Intestines.	Tongue.	Salivary gland.	Pancreas.	Lung.	Trachea.	Thyroid.	Thymus.	Heart.	Arteries.	Muscles.	Skin.	Nervous system.	Urinary bladder.	Testicles.	Prostate.	Uterus and ovary.
1	30	M.	5	23	20 cc. serum 5th day.	Formalin.	−	+		+	−	−	−	−	+								+		−						
2*	59	"	6	18		"	+++	+		+++	+	+++	+					+													
3	57	"	6	4	20 cc. serum 4th and 5th days. Salvarsan.	"	−	−		+	+	−	−		+			+					+								
4†	48	"	6			"	+	+					−																		
5*	45	"	7	25		"	+	+		+	+		+	+	+	+		+	+	+			+	+	+		+	+	+	
6	47	"	7			"	+	+		+	+		+	+	+			+		+			+	+			+			
7	28	"	8	22		"	−	+		+	+		−	+	+			−	−	−	+		+	+	−		−			
8	44	F.	8	3	60 cc. serum 4th day; 20 cc. 5th day.	"	−	+		+	+	−	−	+	+	+		−			+		+	+	−		−			+
9	34	M.	8	20		"	+	+		+	+		+	−					−				+								
10	49	"	8			"	+	+		+					−				−				+								
11	56	F.	8	12	20 cc. serum 6th and 7th days.	"	−	−		+	+	+	−	−	−		−	−	−	−	−		+	−	−		−			−	
12		M.	8			"	+	+		+																				
13	59	"	9	17	40 cc. serum 6th and 7th days.	"	+	+		+	+	+	−	+	−		+	−	−	−	+		+	+	−		+			+	
14	22	"	9	16		Orth's fluid.	+	+		+	+		−	−	−			+	+						+	+					
15‡	70	F.	9	19		"	−	−			++			−				−	−												

| | | |

No.	Age.	Sex.	Day of illness.	Hours until autopsy.	Treatment.	Fixing fluid.	Liver.		Gall bladder and ducts.	Kidney.		Suprarenals.	Spleen.	Lymph glands.	Intestines.	Tongue.	Salivary gland.	Pancreas.	Lung.	Trachea.	Thyroid.	Thymus.	Heart.	Arteries.	Muscles.	Skin.	Nervous system.	Urinary bladder.	Testicles.	Prostate.	Uterus and ovary.
							Extracellular.	Intracellular.		Interstices.	Tubules.																				
33	24	M.	16	12	20 cc. serum 9th day; **40** cc. 12th day.	Formalin.	−	−		−	+	−	−	−	−			−	−				−	−	−		−		−	+	
34	64	"	16	5	40 cc. serum 10th day.	"	−	−		−	++	−	−	−	−			−	−				+	−	−		−		−	+	
35*	35	"	17	2		"	−	−		−	+	−	−	−	−			−	−				+	+	−		−		−		
36*	41	"	18	2		"	−	−		−	+++	−	−	−	−			−	−				+	+	−		−		−		
37	25	"	18	4	**40** cc. serum 12th day; **40** cc. 13th–15th days.	"	−	−		−	++	+	−	−	−			−	−				+	+	−		−		−		
38	57	"	20	19	20 cc. serum 11th day; 40 cc. 12th–15th days.	"	−	−		−	+	−	−	−	−			−	−				+	−	−		−		−		
39‡	39	"	27	18	**20** cc. serum 5th day.	"	−	−		+	+	−	−	−	−			−	−				−	−	−		−		−		
40	32	"	35	13	**60** cc. serum 14th–16th days; neo-salvarsan 7th day.	"	−	−		−	++	−	−	−	−			−	−		+		−	+	−		−		−		
41		"	50			"	−	−		−	+		−					−	−				+	−	−		−				
42	51	"	55	16	20 cc. serum 5th and 6th days.	"	−	−		−	+		−					−	−				−	−	−		−		−		
43‡	32	"	?			"	−	−		−	+		−					−	−				+								

In the organs, with the exception of the kidneys and the heart, complete destruction of spirochetes has taken place. The kidneys, on the other hand, show the organisms constantly, and particularly in the tubules. In Case 42 they could be demonstrated in the urine up to the 55th day. The experimental animals treated with immune serum and salvarsan also harbored spirochetes in their kidneys for a long time after recovery. It was observed in white rats, mice, rabbits, and occasionally in guinea pigs, that the spirochetes lodge solely in the kidneys.[5] The relationship of the spirochetes to the kidneys is noteworthy in that the immune bodies are ineffectual against the organisms contained in the kidney tubules. The spirochetes found occasionally in the cardiac muscles are mostly degenerated.

Summarized briefly, the distribution of the spirochetes in the various organs of the human body differs according to the degree of development of the immune bodies. The spirochetes disappear first from the liver and suprarenals, but remain for some time in the muscles, prostate, thymus, appendix, testicles, epididymis, etc. In the kidneys they can be found for a long time. Their mode of disappearance would seem to indicate distribution varying with the different stages of the disease. And not alone the general distribution, but the local position of the spirochetes changes with the different stages. In the early stage, they are located mainly extracellularly, in the interstices; in the later stage, owing to the development of the immune bodies, in the blood, in the main intracellularly, and within the kidney tubules.

Distribution of Spirochæta icterohæmorrhagiæ in the Tissues.

In the first stage of Weil's disease the organisms are located extracellularly, in the tissues.

Spirochetes are rarely found in the epithelial cells, the kidney tubules, and other glands. They are lodged chiefly in the interstitial spaces surrounding the cells, frequently around the periphery of cells. This position is characteristic, and typical in guinea pigs. In man, in whom a development of immune bodies

[5] Drs. Ido, Hoki, Ito, and Wani, after careful examination of the various organs of house rats were able to find *Spirochæta icterohæmorrhagiæ* only in the kidneys. Ido, Y., Hoki, R., Ito, H., and Wani, H., The rat as a carrier of *Spirochæta icterohæmorrhagiæ*, the causative agent of Weil's disease (spirochætosis icterohæmorrhagica), *J. Exp. Med.*, 1917, xxvi, 341.

takes place in the blood, the distribution is somewhat different. Here the spirochetes are more frequently enclosed in the cells and cell tubes, and increasingly so with the progress of the disease. The spirochetes disappear first from the interstitial spaces, remaining for some time longer in the cells and kidney tubules. This phenomenon is less marked in the liver and suprarenals, where the spirochetes are destroyed at an early stage; it is very marked in the kidneys. Spirochetes proliferate in the kidney tubules and are excreted with the urine.

Muscular tissue, such as that of the heart and skeletal muscles, retains the spirochetes for a relatively long time. The organisms are seen to surround the muscle fibers closely, but are rarely found within them, though this may be the case in late stages of the disease. In a patient dying on the 9th day, we observed densely massed spirochetes in degenerated hyaline muscle fibers. It seems as though the spirochetes were able to penetrate more readily degenerated or broken cells than those that are intact. This is also true of epithelial cells. Smooth muscle reacts in the same manner as striated. The muscular tissue of the prostate, urinary bladder, gall bladder, the vessel walls, and the intestinal walls are lodging places during the second stage. On one occasion we observed numerous spirochetes in the medullary substance of the suprarenals, while the cortex was already free from spirochetes. In smooth muscle, the organisms are not located parallel to the muscle fibers, but cross them irregularly. The spirochetes occur but rarely in the interstices of the nervous system.

The connective tissue contains spirochetes in abundance. It is, in fact, a main repository for them. Carried by the blood stream, they reach first the walls of the capillaries, and from there are spread over the surrounding connective tissue. Hence they are to be found in great numbers in the lumen, the walls, and the perivascular connective tissue of the capillaries. In man by the 6th day the spirochetes have left the capillaries, having proceeded by way of the delicate fibrous strands, deep into the parenchyma. The coarse connective tissue shows a sparse distribution. The spirochetes are assembled mostly in the dividing tissue lying between the connective tissue and the parenchyma, or in the narrow interstitial layer of connective tissue. They disappear relatively early from the connective tissue, though scattered specimens may be observed in the coarse connective tissue for a long time. The spirochetes may also be carried by the lymph channels, though it is difficult to demonstrate them in the lymphatic vessels.

The spirochetes are often harbored for some time in homogeneous substances, such as urinary casts, degenerated muscle fiber, in colloidal masses of the thyroid, and other coagulated masses. They seem to show a preference for substances of this kind, where they can be secure against the action of the immune bodies.

Phagocytosis.—In the early stage spirochetes are rarely found enclosed in leukocytes, endothelial cells, or connective tissue cells; but in the course of the

disease they are increasingly found in phagocytes. This observation was confirmed experimentally. Phagocyted spirochetes were found in great numbers following the injection of immune serum into guinea pigs. In man, the phenomenon is not quite so marked, owing perhaps to the fact that the development of the immune bodies is a gradual process, while in the animal large numbers of immune bodies are introduced suddenly. The spirochetes contained in phagocytes are in various stages of degeneration. It would appear as if the spirochetes first degenerate and are then taken up by the phagocytes. Organisms not degenerated but showing regressive changes are sometimes found in phagocytes at the beginning of illness. From this we may assume that they actively penetrate the degenerated phagocytes, and are passively received by the latter.

Distribution of Spirochetes in the Organs.

Liver and Bile Ducts.—In our experimental work with guinea pigs we found that the liver contained the densest distribution of spirochetes, while in man the organisms are rather scattered in that organ. Only in one patient dying on the 6th day and in one of Iida's 8 day cases were we able to demonstrate them numerously. In the densest region, ten to twenty were counted to the optical field. As a rule, the organisms are located extracellularly. Within the lobes they are found close to the fine fibrous strands or between the hepatic cells. In our cases they were associated intimately with markedly developed fibrous strands in the periphery of the acini. In the hepatic cells and the stellated cells of Kupffer they are found but rarely, and then in degenerated condition. The spirochetes located between the hepatic cells are bent and adhere with their ends or bent sides to the cells. At times they are located in almost parallel lines on the periphery, giving the appearance of lying in the intercellular bile duct capillaries, but their demonstration within the capillaries is difficult. Only in one instance were we able to observe a spirochete in a somewhat dilated biliary duct. It is not probable that the spirochetes stand in any close relation to the bile duct capillaries. In man they are also not so closely related to the blood capillaries as in the guinea pigs. They are rarely present in the interstices of the lobes; *i.e.*, in the interlobular connective tissue. They are infrequently found massed about the acini and the bile ducts; they enter often into the wall epithelium of the ducts, but almost never into the lumen. They are also rare in the zone of leukocytic infiltration, as well as in the interstitial blood vessels, and are seldom found in the lumen. The spirochetes in the liver are frequently contained in degenerated cell masses and coagulated homogeneous substances. On the whole, distribution within the liver is irregular. In the course of the disease, the spirochetes in the liver are almost completely destroyed, and those found occasionally enclosed in stellated cells are mostly of the degenerated type.

The large bile ducts and the gall bladder were examined in five patients who died during the second stage of Weil's disease. Very rarely were spirochetes found in the subepithelial fibromuscular layer.

Kidneys.—The kidney is characterized by a more or less dense distribution of spirochetes throughout the course of the disease. We were able to demonstrate them up to the 55th day of illness. In cases of mixed infection treated with immune serum, scattered specimens are found in the kidneys after they have disappeared from other organs. In fact the organisms could be found in varying numbers in the kidneys of all cases.

In earlier stages the spirochetes are present mostly in the interstices, more numerously in the cortex than in the medullary substance. They are found in the fibrous strands (trabeculæ), in the tissue spaces, and in the interstitial cells. They are intimately related to the capillaries. In the kidney tubules they are found surrounding the tunica propria. They are rarely contained in the epithelium and the lumen of the kidney tubules. In the course of the disease part of the spirochetes are gradually destroyed, but even in later stages, numerous organisms can be detected in the tubules. In the convalescent stage, innumerable, thickly grouped spirochetes are found in the tubules. It is evident, therefore, that the spirochetes proliferate in the tubules. The number found in the kidneys does not parallel the course of illness. In a patient dying on the 8th day without complicating symptoms, the organisms were sparse, while in one dying on the 10th day, spirochetes in large numbers were found. The latter case was of interest because the spirochetes were thickly grouped in the interstices. In the densest regions, the organisms covered the whole area of the interstitial space and closely surrounded the tubules. It was possible to observe them by low power of the microscope as black dots or spots. Occasionally one sees a case in which the organisms are found mainly in the tubules.

The spirochetes in the tubules lie in the pockets of the lumen, close to the epithelium. They are to be found in the detritus mass or in urinary casts, seldom in epithelium, sometimes in vascular desquamated epithelium.

The spirochetes in the tubules are frequently located in the border zone between the cortex and the medullary substance, the free organisms in the convoluted tubules, and those enclosed in casts in the straight tubules.

In man the spirochetes were never observed in the glomeruli, though in guinea pigs they were found there in sparse numbers. We may conclude, therefore, that the spirochetes in the tubules are not flooded out by means of the glomeruli, but rather that those contained in the interstices migrate through the walls of the tubules directly into the lumen, where they are preserved and may proliferate.

Suprarenals.—The suprarenals, like the kidneys, are characterized in the guinea pig by a dense distribution of spirochetes. Many were seen in a patient dying on the 6th day. They were contained in the interstices, combined with the interlobular fibrillæ of the upper cortex layer. In the cortex cells and their sheaths, they were scarce. They were irregularly distributed, and were found mainly in the subcapsulary layer, almost never in the medullary, and the deep cortex layer. After the 8th day, only a few degenerated residual forms are present. The latter lodge in the cortical layer and in the interstitial cells. Occa-

sionally spirochetes are located extracellularly, between the cortical cells and in the interstices. In a patient dying on the 8th day we observed a number of intact specimens in the medullary muscles.

Spleen, Lymph Glands, and Bone Marrow.—The hematopoietic organs showed throughout a sparse distribution of spirochetes. In the spleen the organisms are found at an early stage in the pulp, trabeculæ, and capsular tissue, very rarely in the Malpighian bodies. In the experimental animals it was also difficult to find spirochetes in the Malpighian bodies, particularly in the center. In the pulp tissue, the spirochetes are found mainly in the lattice fibers and the fibrous strands of the sinus and vessel walls. They are likewise present in the pulp cells and in the phagocytes. The phagocyted spirochetes are mostly degenerated. On the other hand, the spirochetes found in the trabeculæ and other coarse fibrous tissue are relatively well preserved. Spirochetes are sometimes found in the walls of large arteries and in their vicinity.

Further on in the disease, spirochetes are rarely present in the trabeculæ, in coagulated masses, and in phagocytes. They are then mostly degenerated in type, though sometimes in good condition in the trabeculæ.

Numerous organisms are contained in swollen lymph glands at the beginning of illness. At first, they are present in the regions of the lymph vessels and sinuses. On the 2nd and 3rd day of illness they appear deeply seated in the fibrous tissue of the parenchyma. The vessel walls, the trabeculæ, and the perivascular tissue also show them. Spirochetes are found extracellularly in the peripheral zones of the follicles. Phagocyted spirochetes are rare. On the 3rd and 4th day we find them numerously in the lumen of the vessels. It would seem, therefore, that the organisms gain access to the lymph channels through the local lymph glands. From there they go into the parenchyma and blood vessels. This phenomenon was confirmed on animals. After the 7th day, it is difficult to find spirochetes even in swollen lymph glands. In the mesenteric glands, they are more numerous, but degenerated and contained mostly in phagocytes. They are rare in fibrous strands. Later on, only scattered residual forms are seen in phagocytes and fibrous tissue.

The conditions in the lymphoid tissue of the various organs and the tonsils resemble those of the lymph glands.

The bone marrow, particularly that of the tibia, was studied in a few cases. In a patient dying in the second stage, no spirochetes were found. In guinea pigs, on the other hand, spirochetes were as numerous in the bone marrow as in the blood.

Digestive Tract and Pancreas.—In guinea pigs closely packed spirochetes are found in the subepithelial mucosa of the intestinal wall. In man their distribution in the intestinal wall was rather scanty on the 7th day and throughout the second stage. In the appendix wall and that of the large intestine and stomach, the spirochetes are more numerous at this stage. They are scattered throughout the mucosa, somewhat more numerous in the submucosa and muscular layer.

They are found free in the tunica propria or in phagocytes, and are often lodged close to the epithelial layer, rarely in the layer itself. They are present for a long time in the muscular layer, and often in considerable numbers. In the convalescent stage, spirochetes do not occur in the intestinal wall; but occasionally a few are found in the intestinal crypts and lumen, though we are unable to say definitely that the organisms were *Spirochæta icterohæmorrhagiæ*, as they were very closely grouped and well formed, which are not characteristics of this spirochete. Moreover, in the silver preparation they did not appear granular, and similar organisms are often found in healthy persons. Dark-field illumination is required to decide this point.

Judging by analogy with the kidneys, it may be supposed that the *Spirochæta icterohæmorrhagiæ* wander out into the intestinal lumen. Infection experiments carried out on guinea pigs with intestinal contents were occasionally positive. In some cases, the organisms may be identified with certainty in the intestinal lumen. These spirochetes are clearly granulated and are located in desquamated epithelium and detritus masses.

The esophagus, tongue, and salivary glands also harbor the spirochetes in varying numbers. As a rule, they lodge in the interstices early in the disease, and later on in the cells.

The pancreas showed spirochetes on the 6th or 7th day. In the densest region a number of specimens appeared in the optical field. They occur mainly in the interstices, closely surrounding the body of the gland and the excretory duct, rarely in the lumen and in the epithelial cells. Later in the disease only a few degenerated forms were found, mostly in the cells.

Respiratory Organs, Thyroid, and Thymus.—The lungs contain but few spirochetes, only a small number being found in the alveoli on the 6th day. They were enclosed in desquamated epithelium, blood, and blood coagulum. Later on, it is almost impossible to find them; very rarely one finds degenerated forms in the alveolar epithelium and coagulated masses.

In the trachea spirochetes were present in the interstices on the 7th day in considerable numbers. In another individual, dying on the 8th day, they were scattered.

A few spirochetes were present in the interstices and capsule of the thyroid in a patient who died on the 6th day. At a later stage they are rarely found in the colloidal masses.

The thymus, which was examined in two cases, showed a relatively thick distribution of spirochetes. In a patient dying on the 10th day, they were found rather plentifully in the capsule and septum tissue, and also enclosed in thymus cells. The other patient, who had died on the 12th day, had received serum treatment. Here also numerous organisms were present, mainly degenerated forms, located extracellularly.

Circulatory System.—In the search for spirochetes in man, particular attention has been paid to the heart, for the number there is much greater than in guinea

pigs. On the 7th day of illness and throughout the second stage, spirochetes are more or less numerous; a few were seen in an optical field. Early in the disease they are found in the epicardium and endocardium, and most constantly in the muscular layer. They occur first in the interstices, later on in muscle cells. In the interstices they are found in the perimysium, closely surrounding the muscle cells, in the neighborhood of the capillaries, in coagulated venous blood, and in phagocytes. Those enclosed in cells are found in the periphery and in the centers of muscle cells, independent of the striation. On the whole, they are more numerous in the right side of the heart than the left. The sinoauricular region also showed a dense distribution. During convalescence spirochetes are found here and there, mostly degenerated.

In the arterial walls of the aorta, carotids, brachials, and radials, spirochetes are present more or less constantly during the second stage, chiefly in the media, seldom in the adventitia. They are not numerous. In the lumen of the brachial artery, we observed a single specimen in a patient dying on the 9th day. This was located in coagulated blood near the wall.

Skeletal Muscles and Skin.—The skeletal muscles showed on the 7th day (second stage) a relatively dense distribution, particularly in the calf muscle and rectus abdominis, where the histological changes were most marked. On the 9th day a few specimens were seen. The spirochetes found in muscles occur as a rule in the interstices, *i.e.*, the perimysium, and they surround the muscle fibers closely. Only once did we find thickly grouped spirochetes in degenerated hyaline muscle fibers. At a later stage the organisms are rarely seen in muscle fiber.

The outer skin rarely shows spirochetes. We could discover them only in two out of four cases—in the one contained in prickle cells, in the other in the corium. None were found in the lumen of the sweat and sebaceous glands.

Nervous System.—At an early stage (the 6th day) spirochetes were found in the interstices and the spinal meninges. In rare instances they were present in the interstices of the central and peripheral nervous systems. In the gray and white substance of the brain and spinal cord, degenerated forms were found on a few occasions. Once they were present in the neuroglia.

Urinary Bladder and Genital Organs.—On the 7th day, and during the second stage, a large number of spirochetes were present, particularly in the muscular layer of the bladder. In the testicles and epididymis, well preserved specimens were seen on the 6th and 7th days (second stage), in the fibromuscular tissue, but seldom in the tubules. The prostate shows an unusual condition. On the 7th day (second stage), numerous spirochetes were found in the fibromuscular tissue. The muscular layer of the uterus and the fibrous tissue of the ovaries may show scattered spirochetes, those in the uterus being in well preserved condition.

Factors Influencing the Distribution of Spirochæta icterohæmorrhagiæ.

Severity of Illness.—Those cases of Weil's disease, in which the pathological changes are particularly marked, show in the main the densest distribution in the various organs. In guinea pigs also the distribution of spirochetes is more or less paralleled by the degree of icterus and hemorrhage.

Mixed Infections and Complications.—Suppurative or septic infections, which often accompany Weil's disease, greatly influence the distribution; the spirochetes in the organs thus become reduced in number. They are often absent from all the organs, except the kidneys and the cardiac muscles. Cirrhosis of the liver does not seem to affect the distribution.

Serum and Salvarsan Treatments.—The spirocheticidal action of immune serum has been previously described.[6] The statements then made are supported by our postmortem findings. Very few spirochetes are discovered in the organs, with the exception of the kidneys, where they remain for a long time. The spirochetes in other organs had evidently been destroyed.

Types of Spirochete in Human Tissues.

In comparison with those found in the experimental guinea pigs the spirochetes in man are irregular in type. They show greater rigidity than those in the animals. They are atrophic and shrunken, and of varying thickness. The edges are not smooth and the curves are irregular; many small waves are observed. At one or both ends, or in the middle, a circumscribed thickening may be present. Sometimes one-half of the spirochete differs in thickness from the other. The circumscribed thickening can be distinguished from the bud, as the latter is more markedly granular. The spirochetes may appear as large, irregularly distributed chains of granules. One end often shows an enlarged, granular bud. This rare form was distributed in a number of organs. It seems to arise through a loosening and

[6] Inada, R., Ido, Y., Hoki, R., Ito, H., and Wani, H., The serum treatment of Weil's disease (spirochætosis icterohæmorrhagica), *J. Exp. Med.*, 1916, xxiv, 485.

enlargement of some of the granules of the spirochetes. Abortive forms (rod-like, comma-shaped, or bent) arise through the breaking off of parts of the spirochete. One end may give the appearance of having dissolved. The large bud may be present at one end of a markedly shrunken spirochete. Such deformed types are often found in phagocytes.

In the group, where the spirochetes are closely assembled, many small granules or rings are seen; these stain black when impregnated with silver. The granules are globoid or irregularly formed, and are from two to five times as thick as the spirochetes. They are detached, fused buds or broken parts of spirochetes. The rings referred to above are round or somewhat irregular, also from two to five times as large as the thickness of the spirochete. Their origin is identical with that of the granules. On the other hand, the ring type of spirochete which has already been described, is large and irregular, and can be readily distinguished from the rings referred to above.

We are led to believe that these forms are degenerated products, caused by the action of the immune bodies. This point requires further investigation.

In closing we desire to express our thanks to the Director, Dr. Ryokichi Inada, for his assistance in the work, and to Dr. H. Nakayama of the Pathological Institute of Kyushu University for placing at our disposal material for investigation.

THE RAT AS A CARRIER OF SPIROCHÆTA ICTERO-HÆMORRHAGIÆ, THE CAUSATIVE AGENT OF WEIL'S DISEASE (SPIROCHÆTOSIS ICTEROHÆMORRHAGICA).*

By YUTAKA IDO, M.D., ROKURO HOKI, M.D., HIROSHI ITO, M.D., and HIDETSUNE WANI, M.D.

(*From the First Medical Clinic of the Imperial University in Kyushu, Fukuoka, Japan.*)

(Received for publication, February 20, 1917.)

In a previous communication[1] on the prophylaxis of spirochætosis icterohæmorrhagica, we discussed briefly the excretion and means of invasion of the causative agent. It was clear that soil and water, particularly stagnant water, are related to the infection, but where the spirochetes have their habitation outside of the human body and proliferate until they again attack man remained a difficult problem of prophylaxis.

When Inada at the annual meeting of the Kitasato Institute for Infectious Diseases, in the spring of 1915, gave a comprehensive report of studies made on spirochætosis icterohæmorrhagica in his clinic, Miyajima called attention to the fact that in his investigations on tsutsugamushi, he had found on several occasions spirochetes resembling *Spirochæta icterohæmorrhagiæ* in the kidneys of the field mouse, *Microtus montebelloi*. On the basis of these findings, we conducted during the following year an investigation on twenty-two house and roof rats, *Epymis alexandrinus* and *Epymis norvegicus*. We discovered on one occasion in the kidney of one of the animals a specimen of *Spirochæta icterohæmorrhagiæ*. There was at that time doubt in our minds whether the organism in question had actually resided in the kidney or was a contamination introduced from the outside,

* Published in *Tokyo Iji-Shuho*, July, 1916, Nos. 1978, 1979.

[1] Ido, Y., Hoki, R., Ito, H., and Wani, H., The prophylaxis of Weil's disease (spirochætosis icterohæmorrhagica), *J. Exp. Med.*, 1916, xxiv, 471.

inasmuch as we were working with *Spirochæta icterohæmorrhagiæ*. The problem was then left for future investigation.

In the spring of 1916, Miyajima again reported that he had found in *Microtus montebelloi* spirochetes resembling *Spirochæta icterohæmorrhagiæ*, which injected into guinea pigs produced fever and hemorrhage, and after a number of generations, icterus.[2] As the immune serum of *Spirochæta icterohæmorrhagiæ* was capable of destroying the organisms in question, he concluded that they were identical with *Spirochæta icterohæmorrhagiæ*.

From our clinical experience we had already surmised the relation of the rat to the infection in man. Cooks working in kitchens frequented by rats often became ill with spirochætosis icterohæmorrhagica. At the beginning of the year we observed two typical cases following the bite of rats. We were led to the conclusion that the rat plays an important part in the transmission of the infection, and that the spirochete previously found by us in the kidney of a rat was not a contamination, but came from that organ. On the basis of this assumption we undertook an investigation of the house and roof rats in the city of Fukuoka and its vicinity.

EXPERIMENTAL.

The rats examined were *Mus alexandrinus* and *Mus decumanus*. We were able to find virulent *Spirochæta icterohæmorrhagiæ* in the kidneys, in 40.2 per cent out of 149 *Mus decumanus*, and in 0.8 per cent of 24 *Mus alexandrinus*.[3] The morphological examinations and

[2] The findings of the Japanese workers have been confirmed in Europe by Dr. Stokes, who conducted an investigation of cases of Weil's disease occurring in the British army. He was able to demonstrate *Spirochæta icterohæmorrhagiæ* in the kidneys of field rats, and infected guinea pigs with the organisms (Stokes, A., Ryle, J. A., and Tytler, W. H., Weil's disease (spirochætosis icterohæmorrhagica) in the British Army in Flanders, *Lancet*, 1917, i, 142). In the United States, Dr. Noguchi has confirmed the work by his finding of *Spirochæta icterohæmorrhagiæ* in domestic wild rats (Noguchi, H., *Spirochæta icterohæmorrhagiæ* in American wild rats, and its relation to the Japanese and European strains, *J. Exp. Med.*, 1917, xxv, 755).

[3] The determination of the animal species was made by Mr. Namiye, assistant in the Zoological Institute of the Imperial University at Tokyo, to whom we desire herewith to express our thanks.

specific differences in the immune serum proved that the spirochete found by us in the kidneys of house and wild rats is identical with *Spirochæta icterohæmorrhagiæ*.

We also examined six specimens of field mice.[4] In one instance we produced by intraperitoneal injection of mouse kidney emulsion, icterus and hemorrhages in the guinea pig, and identified numerous *Spirochæta icterohæmorrhagiæ* in the liver, though we found none by dark-field illumination in fresh kidney preparations of the field mouse. In five other cases, we could find no spirochetes, either by dark-field illumination or through intraperitoneal injection of kidney emulsion.

In our experiments with rats we employed the following method. A rat was permitted to bite a guinea pig in the hind leg. The rat was then killed, and a search was made by dark-field illumination for spirochetes in the blood, liver, and kidneys. When no organisms were found in this way, we injected intraperitoneally into the guinea pigs the blood, urine, liver, and kidney emulsion of the rat, awaiting the development of icterus and hemorrhages. As a rule, we examined three preparations by dark-field illumination, and if no organisms were found, we considered the result negative.

Experiment 1. Kidneys.—The number of rats employed was 92. In 26 of the animals, or 28.3 per cent, spirochetes could be demonstrated in the kidneys. We injected kidney emulsion from 8 of the 26 animals intraperitoneally into guinea pigs. 7 of these died on the 8th to the 13th day with marked icterus and hemorrhages; numerous organisms were found in their blood and liver. From 59 animals showing microscopically no organisms, we prepared kidney emulsion, which we injected intraperitoneally into guinea pigs; 5 (8.5 per cent) animals died with typical symptoms on the 8th to the 11th day. The number of spirochetes in the kidneys varied between one to a preparation and fifteen to sixteen in an optical field. Occasionally we found a tuft of spirochetes. All specimens showed brisk movements.

Experiment 2. Urine.—We examined the urine of 71 rats. In 22, or 31 per cent, spirochetes were present. In 19 rats, in which we found the organisms in the kidneys, they were contained also in the urine. In 3 out of 52 in which we were unable to demonstrate spirochetes in the kidneys, we discovered them in the urine. The urine sediment of 2 rats, in which no spirochetes had been found by either method, was injected intraperitoneally into guinea pigs. One

[4] These mice were furnished us through the kindness of Dr. Miyakawas.

of the experimental animals died with typical symptoms on the 17th day. To test the virulence of the spirochetes, we then injected 0.1 to 0.2 cc. of urine containing spirochetes, from 5 rats, intraperitoneally into 5 guinea pigs. The animals died with marked icterus and hemorrhages 8 to 10 days later, thus proving a high degree of virulence for the organisms.

The spirochetes excreted in the urine appear for the most part in the nubecula, though some are found free. Their number fluctuates from fifteen to sixteen specimens in one nubecula to one or two in a preparation. The spirochetes in the nubecula are usually motionless, while the freely floating spirochetes make brisk movements. Occasionally degenerative forms are seen.

Experiment 3. Blood and Liver.—We examined the blood of 64 rats by dark-field illumination. In 20 of these we had found spirochetes in the kidneys and the urine, but we were unable to find them in the blood in a single instance. The intraperitoneal injections of guinea pigs with the blood of 6 rats, in which organisms were found in the kidneys, proved negative, as well as five blood injections from animals having no spirochetes in their kidneys. Similar experiments were conducted with liver emulsion and intestinal contents. We examined the livers of 62 rats, and the large intestines of 10. All these experiments were negative.

Experiment 4. Rat Bite.—As we had observed two cases of typical spirochætosis icterohæmorrhagica following the bite of a rat, we proceeded to reproduce the conditions experimentally. Rats were permitted to bite guinea pigs in the leg. Of 50 experiments thus made, only one guinea pig died of icterus and hemorrhages on the 11th day following the bite. In this case, numerous *Spirochæta icterohæmorrhagiæ* were found in the blood and the liver. In two guinea pigs we observed in the blood another form of spirochete which Futaki and Ishiwara, with their associates, have assigned as the cause of rat-bite fever. It may be mentioned in passing that Drs. Kaneko and Okuda[5] of our clinic were able to confirm the view of the other Japanese investigators in this respect by finding the spirochetes in the kidneys of a patient dying from rat-bite fever. By our finding of spirochetolytic and spirocheticidal immune bodies in the serum of individuals who had recovered from rat-bite fever, we were able to affirm that the above mentioned spirochete is the causative agent of rat-bite fever.

Mizukuchi reported before the Pathological Anatomical Congress held in Tokyo in April, 1916, an interesting fact which grew out of an experimental investigation of rat-bite fever. He permitted guinea

[5] Kaneko, R., and Okuda, K., The distribution in the human body of *Spirochæta icterohæmorrhagiæ*, *J. Exp. Med.* 1917, xxvi, 325.

pigs to bite rats, and certain of the animals died later, as the result of a peculiar icteric and hemorrhagic condition. In the liver of these animals he found a spirochete which, according to our view, is identical with *Spirochæta icterohæmorrhagiæ*. Mizukuchi believed the spirochete to be the cause of rat-bite fever, a conclusion with which we cannot agree. It is highly probable that the microorganism was *Spirochæta icterohæmorrhagiæ*, and that it was communicated to the guinea pigs in the same manner as in our experiments.

The manner in which *Spirochæta icterohæmorrhagiæ* is conveyed by the bite of a rat to man or the guinea pig is not yet clear. As no spirochetes were found either in the mouth or the blood of the rat, it is not likely that they are carried directly through biting. It may be assumed, however, that they are conveyed indirectly from rat urine, with which the mouth of the rat may be contaminated, into the wound created by the bite of the rat.

As stated above, the spirochetes found in the kidneys and the urine of rats resemble in form and movement *Spirochæta icterohæmorrhagiæ*. When injected intraperitoneally into the guinea pig, the animal succumbs after a time, with icterus and hemorrhages, symptoms which are identical with those of spirochætosis icterohæmorrhagica. These facts seem to prove without a doubt that the spirochete in question is identical with *Spirochæta icterohæmorrhagiæ*, the causative agent of Weil's disease, but further experiments to confirm this point are cited below.

Action of Spirochæta icterohæmorrhagiæ Immune Serum on Spirochetes Found in Rats.

For Pfeiffer's tests we used guinea pig liver emulsion rich in spirochetes, and immune horse serum of *Spirochæta icterohæmorrhagiæ*. Control experiments were also carried out. These were made (1) with rat spirochetes, *Spirochæta icterohæmorrhagiæ*, and isotonic salt solution; and (2) with *Spirochæta icterohæmorrhagiæ* and its immune horse serum. The results are shown in Table I.

As shown in the table, no spirochetes were contained in the peritoneal fluid in from 30 minutes to 2 hours after injection in the main experiments conducted with rat spirochetes and immune horse serum,

TABLE I.

Results by Pfeiffer's Method with Rat Spirochetes and Immune Horse Serum of Spirochæta icterohæmorrhagiæ.

Animal No.	Case No.	Intraperitoneal injections into guinea pigs.					No. of spirochetes in the peritoneal fluid.		Course of experimental animal.	Autopsy.	Spirochetes in liver.	Results.
		Spirochetes.			Serum.							
		Generation.	No.	Amount injected.	Type.	Amount injected.	After 30 min.	After 2 hrs.				
				cc.		cc.						
1	219	R 16/1 III Rat spirochetes.	10 in one field.	1	Horse serum.	1	2 in one preparation.	0 in one preparation.	Well for 1 mo.			+
2	220	" " "	10 " "	1	" "	1	0 " " "	" " " "	" " 1 "			+
		Control experiments.										
3	217	R 16/1 III Rat spirochetes.	10 in one field.	1	Sodium chloride.	1	3–6 in one field.	1–2 in field, lively.	Died 5th day, icterus and hemorrhage.	+	Numerous.	
4	218	Pure culture.	10 " "	1	" "	1	3–6 " " "	1–2 " "	" 5th " " " "	+	"	
5	221	" "	10 " "	1	Horse serum.	1	1 in preparation, lively.	None in preparation.	Well for over 1 mo.			+

and in an experiment made with *Spirochæta icterohæmorrhagiæ* and immune horse serum, while numerous spirochetes were found in the control experiments conducted with isotonic salt solution. These tests show that the serum of horses immunized with *Spirochæta icterohæmorrhagiæ* has a spirocheticidal and spirochetolytic action upon the spirochetes of the rat.

Experiments to Determine Whether Goat Serum Obtained by Immunizing with Rat Spirochetes Will Destroy Spirochæta icterohæmorrhagiæ.

We immunized a number of goats with a pure culture of the rat spirochete or with liver emulsion containing the organism, and were able to obtain an effective serum. We then made Pfeiffer's tests with the goat serum and *Spirochæta icterohæmorrhagiæ* and the rat spirochete in the same order as the previous experiments. Table II shows the results.

The result of these experiments shows that the goat serum obtained by immunizing with rat spirochetes is capable of destroying *Spirochæta icterohæmorrhagiæ*.

On the basis of these experiments, we may conclude that the spirochetes found in the kidneys of house and wild rats are identical with Inada and Ido's *Spirochæta icterohæmorrhagiæ*.

We examined the kidneys of 6 field mice and injected their kidney emulsion into the peritoneal cavity of guinea pigs. One of the animals died on the 25th day of icterus and hemorrhages, but here the period of incubation was too long, and the hemorrhages were insignificant. As up to the present we have not observed stall infection among the guinea pigs, we must conclude that though the field mouse harbors our spirochetes in the kidneys, they are not of great virulence in that animal. Miyajima's findings also are in accord with our observations, for he observed in the kidneys of field mice spirochetes which appeared to be far less virulent than those observed by us in the kidneys of house and wild rats.

TABLE II.

Pfeiffer's Tests Made with Spirochæta icterohæmorrhagiæ and Immune Goat Serum from Rat Spirochetes.

Animal No.	Case No.	Intraperitoneal injections into guinea pigs. Spirochetes. Generation.	No.	Amount injected.	Serum. Type.	Amount injected.	No. of spirochetes in the peritoneal fluid. After 30 min.	After 2 hrs.	Course of experimental animal.	Autopsy.	Spirochetes in liver	Results.
				cc.		cc.						
1	223	R 30/1 IX Rat spirochetes.	10 in one field.	1	Goat.	1	0 in two preparations.	0 in two preparations.	Well for over 1 mo.			+
2	224	B.-C. IX 682 *S. icterohæmorrhagiæ.*	10 " " "	1	"	1	0 " " "	0 " " "	" " " 1 "			+
3	225	" " "	10 " " "	1	"	1	1–2 in specimen,* moving slowly.	0 " " "	" " " 1 "			+
		Control experiments.										
4	226	B.-C. IX 682 *S. icterohæmorrhagiæ.*	10 in one field.	1	Sodium chloride.	1	2–3 in one field, lively.	1 in one field, lively.	Died 5th day, icterus and hemorrhages.	+	Numerous.	
5	229	R 30/1 IX Rat spirochetes.	10 " " "	1	" "	1	2–3 " " "	1 " " " "	" 5th " " " "	+	"	

* Specimen covered about 70 optical fields, Leitz oc. 3, $\frac{1}{12}$ oil immersion.

DISCUSSION.

1. In 34 out of 92 cases, or 37 per cent, spirochetes were present in the kidneys or in the urine, as demonstrated directly by dark-field illumination and indirectly by inoculation.

2. The form, movement, virulence, and immune serum prove that our spirochete is identical with *Spirochæta icterohæmorrhagiæ*. The organisms cannot be demonstrated in the blood and the liver, but in the urine of rats harboring *Spirochæta icterohæmorrhagiæ* in the kidneys, they are present without exception.

3. Urine containing spirochetes, even in small amounts (0.1 to 0.2 cc.) infects guinea pigs when injected intraperitoneally.

4. *Spirochæta icterohæmorrhagiæ* are rarely conveyed directly to the guinea pig by the bite of the rat.

5. In Japan, the rat is undoubtedly a carrier of the causative agent of spirochætosis icterohæmorrhagica. *Mus decumanus* was found to be a carrier in 40.2 per cent of 149 cases, *Mus alexandrinus* in 0.8 per cent of 24 cases.

Whether in addition to *Epymis norvegicus* and *alexandrinus* and *Microtus montebelloi* there are still other carriers of Weil's disease is not known.

The Transmission of Spirochæta icterohæmorrhagiæ Infection by the Rat and the Soil.

It is noteworthy that our spirochete appeared only in the kidneys of rats—not in their blood and liver. This peculiarity of distribution is observed also in man during the convalescent stage of Weil's disease and in the guinea pigs treated with immune serum. As already stated, when the antibodies have been fully developed, the spirochete remains only in the kidneys. As, however, the rats examined by us were apparently in a good state of health, notwithstanding the numerous spirochetes found in their kidneys, we may assume that the organisms do not cause any, or only a slight degree of illness. The probability is that, entering the animal by the mouth or skin, after a time they find their way into the kidneys.

We conducted a number of experiments on mice and white rats, injecting 14 mice with 0.5 cc. of blood or liver emulsion from an

infected guinea pig. Four of the animals died with icterus, the others remaining well. Dr. Kaneko found spirochetes only in the kidneys. Of four white rats, one died of icterus; here also spirochetes were found only in the kidneys. It is possible that ordinary rats possess a greater degree of resistance to the infection than mice and white rats.

The behavior of the spirochete within the rat is open for further study, but we know that the rats harboring spirochetes always excrete them in the urine. The organisms thus find their way to the ground, where they may infect other rats as opportunity offers. In all probability they are disseminated by means of rats, the soil and the animals forming a circle of habitation for the spirochetes. It happens rarely that human beings are infected directly through the bite of rats, the infection being usually transmitted from the soil, where evidently the excreted spirochetes lodge and thrive. On these grounds we can explain the epidemics of spirochætosis icterohæmorrhagica which occur in coal mines and among the farmers in the vicinity. Rats are constant tenants of the mines, and it is known that the miners go barefoot. A similar statement may be made concerning the transmission of Weil's disease on the battle-fields of Europe. There the rats living in the trenches infect the soldiers.

It has been stated that the spirochetes of the field mice are less virulent than those of house and wild rats. This fact does not seem to harmonize with the infection of farmers in the fields; but on the other hand, it must be mentioned that wild rats are also found in the fields to some extent. It was certainly very striking that cooks and maids working in kitchens showed so high a percentage of spirochætosis icterohæmorrhagica lesions. Of 84 patients admitted to our clinic, 23 were in occupations which subjected them to contact with rats; *i.e.*, 8 were cooks, 6 maids, 3 pastry cooks, 3 "kamaboko" (bone meal) manufacturers, 2 vegetable dealers, and 1 fish dealer. The clinical observations alone made it probable that there was some connection between the rat and spirochætosis icterohæmorrhagica, though cases in which the infection was traced directly to the bite of a rat are rare. Brief protocols of the histories of two such patients are given below.

Case 1.—Male, age 26; riksha man. Admitted Nov. 24, 1915. On Nov. 11 was bitten by a rat in the little toe of the left foot. The wound bled and was painful, but improved. After a few days, the patient was quite well. On Nov. 17 (7 days after the rat bite) he became ill, with marked general weakness, chills, headache, and pain in thighs. Typical spirochætosis icterohæmorrhagica developed. The incubation period was 6 days.

Case 2.—Male, age 25; waiter in a restaurant. Admitted July 9, 1915. On June 24 had been bitten by a rat in the left first and middle fingers, which bled profusely. On July 2, 1 p.m., patient suddenly had chills, followed by high fever, marked headache, pain in thighs, and general weakness. Typical spirochætosis icterohæmorrhagica. The incubation period was 8 days.

On classifying the rats in respect to their spirochetal content, and the territory from which they came, we find the results shown in Table III.

TABLE III.

Geographical Distribution of Rats Harboring Spirochetes.

Region where rats were caught.		No.	Cases in which spirochetes were found.	Per cent of positive cases.
Fukuoka and vicinity		52	22	42.3
Coal mines in Province of Fukuoka.	Tagawa	9	5	55.5*
	Hondo	8	2	25.0
	Akaike	5	4	80.0
Naokata, Province of Fukuoka		3	0	0
Otsu, Province of Shiga		6	0	0
Tsuchiura, Province of Ibaraki		1	1	100.0
Mimasaku, Province of Okayama		6	0	0
Tottori, Province of Tottori		1	0	0
Totals		91	34	37.4

* Of the twenty-two cases in this group eleven were positive (50 per cent).

It may be mentioned that Dr. Saito, of the First Medical Clinic, traced *Spirochæta icterohæmorrhagiæ* in the kidneys or urine of 50 per cent of the rats in Kyoto.

As shown in the table, the percentage of rats harboring spirochetes is high in those regions where Weil's disease is prevalent, while it is low in regions free from that disease. But it must be remembered that the number of rats examined from the latter regions was small, and that the difference may be attributable to this cause. Miyajima

reported that he found *Spirochæta icterohæmorrhagiæ* in the field mice of a region free from Weil's disease, and it is possible that the rats of a healthy region may also harbor the spirochetes in their kidneys and excrete them with the urine.

The life conditions of the spirochetes seem to be manifold. It was brought out by epidemiological studies that a certain degree of moisture in the soil and a certain temperature are necessary for proliferation. Damp coal mines particularly are favorable places of infection, while the disease is relatively rare in coal mines that are dry. As far as temperature is concerned the optimum in the cultivation of the spirochetes is 22–25°C. Weil's disease in Japan occurs but rarely in the height of summer and the coldest part of winter, but mostly at the end of spring, in early summer, and particularly in the autumn. In coal mines, which have an even temperature, the disease is equally prevalent at all seasons of the year.

That the soil plays an important part in the life of cholera bacilli was emphasized by Emmerlich; and it is known that not only cholera bacilli, but other bacteria and protozoa as well fail to thrive in an acid soil. Our spirochetes also die in a weakly acid medium. This fact led us to trace a connection between the endemic appearance of spirochætosis icterohæmorrhagica and the composition of the soil. Through the kindness of Dr. Takaishi, a member of the Agricultural Institute, we were able to obtain information concerning the distribution of acid, alkali, and neutral soils in the Province of Fukuoka. Comparing the spread of spirochætosis icterohæmorrhagica with the chemical condition of the soil, we discovered the interesting fact that on the whole, the disease occurs rarely in regions having an acid soil, while it is endemic in alkali and neutral soils. The composition of the water and soil of coal mines was also found to vary, an alkali reaction being obtained from the soil of the Ita, Hondo, and Nishizinmachi coal mines, while the earth of the Miike, Yamano, and Akaike mines showed an acid reaction. In Miike no cases of spirochætosis icterohæmorrhagica are known to occur, while in the course of 18 months over 300 cases occurred in Ita, and in Hondo annually from 80 to 100 are recorded. Although there was a high percentage of rats harboring spirochetes in Akaike (80 per cent), only 7 or 8 cases of the disease occur there per year. In the Yamano mine the yearly number is from 5 to 20 cases.

CONCLUSIONS.

1. On the basis of these findings, we conclude that the extermination of rats and field mice is a highly important prophylactic measure against Weil's disease.

2. The chemical composition of soil and water plays an important part in the development of *Spirochæta icterohæmorrhagiæ*, and consequently in the spread of the disease of which it is the causative agent.

We desire to express to Professor Ryokichi Inada our appreciation of the guidance which he has given to our work.

THE CLINICAL ASPECTS OF SPIROCHÆTOSIS ICTERO-HÆMORRHAGICA OR WEIL'S DISEASE.

By RYOKICHI INADA, M.D.

(From the First Medical Clinic of the Imperial University in Kyushu, Fukuoka, Japan.)

(Received for publication, February 20, 1917.)

Stages of the Disease.

Fiedler[1] has divided the progress of Weil's disease into three stages. According to him, the first 2 or 3 days constitute the initial period. The second stage commences variously from the 3rd to the 6th day following the onset of the disease, and is characterized by icterus, edema of the liver, tumor of the spleen, albuminuria, hemorrhagic diathesis, etc. He classes a defervescent period from the 7th to the 8th day as the third stage, and adds also a stage of convalescence. We believe, however, that a different division of the disease into three periods, *i.e.*, first or febrile stage, second or icteric stage, and a third or convalescent stage, has better justification. As will be explained in detail, each stage has its characteristic features with respect to the behavior of the spirochetes in the blood, the antibodies, the excretion of the organisms with the urine, and their distribution in the organs. According to our view, the first and second stages continue each for about a week; and the convalescent stage begins with the 3rd week of illness, although the boundaries of the different stages are not sharply demarcated. Icterus, the main symptom of the second stage, has its beginning in the middle of the first and reaches its climax in the second period.

Febrile Stage.—This stage continues from the onset of the disease to the 6th or 7th day. The main symptoms, which are initiated with chills or high fever, are intestinal disturbances, headache, cramp-

[1] Fiedler, A., Weitere Mittheilungen über die Weil'sche Krankheit, *Deutsch. Arch. klin. Med.*, 1892, l, 232.

ing muscular pains, marked hyperemia of the conjunctiva bulbi, albuminuria, etc. Death occurs rarely in this stage. The period is characterized by free circulation of the spirochetes in the peripheral blood, although their number may not be great. Blood taken during this period and injected intraperitoneally into guinea pigs produces a typical reaction. The infectivity of the blood decreases gradually, as shown by the 69 infection experiments cited in Table I.

TABLE I.

Infection Experiments with Blood from Weil's Disease. April, 1912, to December, 1915.

Day of illness.	No. of animals injected.	Positive.	Per cent positive.
2	4	4	100.0
3	10	10	100.0
4	13	13	100.0
5	12	11	91.6
6	14	12	85.7
7	8	4	50.0
8	4	0	0
9	1	1	100.0
12	1	0	0
18	1	0	0
19	1	0	0
Total.....	69		

As shown in the table, guinea pigs were in all cases infected in a typical manner when they received intraperitoneally blood drawn from patients during the first 4 days of illness. By the 5th day, the infectivity of the blood is already diminished, one case only out of the 12 proving negative (positive 91.6 per cent). With blood taken on the 6th day the results showed 85.7 per cent positive, and on the 7th day 50 per cent. These findings seem to indicate that with the progress of the disease, the spirochetes disappear gradually from the blood stream, owing to the spirochetolytic and spirocheticidal action upon them of antibodies developed in the blood. We are led to believe that antibodies, though few in number, are present as early as the 5th day, but their number is not sufficient for demonstration by Pfeiffer's method.

No difference is observed in this stage of Weil's disease in the peritoneal fluid obtained after the use of serum of patients and that of healthy persons. The guinea pigs under experimentation die of typical symptoms on the same day as the control animals, or a day later or earlier. Hence the inference of a slight development of antibodies can be made only indirectly from results achieved with the infection experiments.

Spirochetes are excreted with the urine during this stage, and the injection of the urinary sediment produces in guinea pigs a typical infection. It is not as a rule possible, however, to demonstrate the organisms in the urine by dark-field illumination. The distribution of spirochetes in the organs resembles that found in the experimental animals. Numerous spirochetes are seen in the liver.[2]

Second or Icteric Stage.—This stage continues from the 7th or 8th to the 12th or 13th day of illness. Generally it covers a little less than a week. As a rule, the symptoms of the first stage decrease in intensity, and in their place appear icterus, hemorrhagic diathesis, marked general weakness, nervous symptoms, and cardiac weakness. But all these symptoms have their onset during the middle or toward the end of the febrile period, and reach their greatest intensity during the second stage. Death is most prevalent during this period. Of eighteen fatal cases, in sixteen death occurred between the 8th and the 16th day from the onset of the disease.

This stage is characterized by the fact that it is rarely possible to infect guinea pigs by the intraperitoneal injection of patients' blood, only one out of six experiments proving positive. It is evident that the spirochetes have already disappeared from the peripheral blood. Moreover, it is possible to demonstrate antibodies in the blood by Pfeiffer's method. The finding of spirochetes in the peritoneal fluid of the experimental animals by Pfeiffer's method shows that a different condition exists in them from that observed in the control animals. Spirochetolysis is present, and the experimental animals die in a typical manner 4 or 5 days later than the control animals. When on the other hand, the injected human serum contains

[2] Kaneko, R., and Okuda, K., The distribution in the human body of *Spirochæta icterohæmorrhagiæ*, *J. Exp. Med.*, 1917, xxvi, 325.

no antibodies, the experimental animals die on the same day as the control animals, or at the utmost 1 or 2 days earlier or later. A difference of 3 days is rare. Hence we conclude that when the experimental animals die 4 or more days later than the control animals, the serum must contain a certain number of antibodies. Their degree of development at this stage is incomplete, for with complete development of antibodies, the experimental animals would recover from the infection.

In our experiments we employed 1 cc. of serum and 1 cc. of pure culture of the spirochetes, or a liver emulsion containing the organisms; ten spirochetes to an optical field by dark-field illumination (Leitz oc. 3, obj. $\frac{1}{12}$ oil immersion). Injections were made intraperitoneally. The gradual increase in the number of antibodies can be observed by testing, according to Pfeiffer's method, serum obtained during various stages of the disease. The duration of life of the experimental animals lengthens in the course of the disease, and finally with complete development of antibodies, the animals do not become ill at all. Occasionally the antibodies are fully developed as early as the 8th day. We observed two cases of this kind.

In the second stage, the spirochetes are easily demonstrated in the urine by dark-field illumination. On the 10th day of illness it was possible to show them in 17.4 per cent of the cases, with a gradually growing percentage up to 52.2.

Corresponding to the development of the antibodies, the spirochetes disappear first from the blood, and then from the liver. Hence their distribution in the second stage differs from that of the febrile stage.

Third or Convalescent Stage.—This period begins on the 13th or 14th day. The intensity of the icterus characteristic of the second stage then subsides gradually, and anemia and marked emaciation become apparent. This period is characterized by complete development of the antibodies in the blood, the disappearance of spirochetes from the blood, their abundant excretion with the urine, frequent high fever (called relapsing fever by Weil, and after fever by us), and later on, by the excretion of antibodies with the urine. The distribution of the spirochetes in the organs is noteworthy. No organisms are found in the liver and other organs except the kidneys,

where they are always present. They are occasionally found in cardiac muscle.

The percentages of spirochetes excreted with the urine gradually increase and reach their maximum on the 15th or 16th, up to the 23rd or 24th day of illness. By the 19th or 20th day practically all cases show spirochetes in the urine. After the 25th day, the percentages decrease. It was found that twenty-two out of twenty-four patients had ceased to excrete spirochetes after 40 days. One patient excreted them on the 42nd day, and another on the 63rd. The duration of most abundant excretion covers from 3 to 6 days. Comparing the onset of the period of abundant excretion of the spirochetes in the urine with the appearance of the complete antibodies in the blood, we find that the first phenomenon precedes the latter by from 2 to 5 days, though occasionally the conditions may be reversed.

Histologically, the spirochetes are found on the 17th or 18th day only in the kidneys, having disappeared from the other organs with complete development of antibodies in the blood.

Incubation Period.

The period of incubation, according to our computations, varies from 5 to 7 days with skin infection; it is seldom as long as 13 days. In the epidemic of Weil's disease which occurred in Hildesheim, Germany, Hecker and Otto estimated the time of incubation as covering at least a week. Our observations in Japan entirely coincide with this finding, and our conclusions concerning the incubation period are based on the histories of patients following our study of the portal of entry of the *Spirochæta icterohæmorrhagiæ in animals.*

Pathology of the After Fever.

The behavior of the fever during the first stage coincides with that observed in cases of Weil's disease in Europe. During the period of convalescence, frequently there is a recurrence of rather high, remittent fever, which Weil and Fiedler have termed relapsing fever. According to Fiedler, in Europe it occurs in 40 per cent of the cases. In Japan we have observed it in 28.2 per cent of our

patients. So far as the character of this fever is concerned, our interpretation differs from that of Weil and Fiedler, and we have employed the term after fever for the following reasons.

The fever has its onset on the 14th or 15th day, sometimes on the 13th, rarely on the 12th or 16th day. There may be an afebrile interval of from 2 to 10 days following the fever of the first stage. The fever covers a period of from 4 to 20 days. It usually reaches a height of 38–40°C., and the temperature is frequently above that of the first stage. The fever is markedly remittent in character, particularly at its maximum. The temperature rises gradually, remains from 3 to 4 days at its greatest height, and then begins gradually to decline. In fatal cases, there is no after fever.

As indicated above, we cannot agree with the view of Weil and Fiedler that this fever is to be regarded as relapsing in character, on the following grounds.

(*a*) We have never observed a recurrence of the main symptoms; *i.e.*, hyperemia of the conjunctiva bulbi, exacerbation of the icterus, hemorrhagic diathesis, edema of the lymph glands, etc. Notwithstanding the presence of the fever at this time, only the symptoms which usually accompany a rise in temperature are found, such as headache, general weakness, etc.; and although the temperature is high, life is not endangered. The fever of the first stage is accompanied by marked leukocytosis, while this condition is not constant in the later fever.

(*b*) Secondly, all the infection experiments conducted on guinea pigs during this period were negative. With the exception of the kidneys, no spirochetes, or very few, are found in the organs.

(*c*) In the third place, the spirochetolytic and spirocheticidal antibodies are fully developed in the blood at this period. If, on the other hand, spirochetes were to reappear in the blood, we should be justified in regarding this fever as relapsing.

We believe, therefore, that the fever of the third stage is different in character from that of the first.

The pathogenesis of the after fever is not clear. Clinically, no particular changes are observed in the organs, and there are no complications or suppurations. The blood of eight patients which was subjected to careful bacteriological examination was found to be

sterile. Hence we are unable to trace the origin of the fever to a secondary infection. It appears that the after fever coincides with the presence of the antibodies in the blood and the abundant excretion of spirochetes in the urine. Furthermore, the curve of the serum-treated guinea pigs which survived resembled closely the fever curve of patients in the third stage of illness.

It is our belief that this fever is to be regarded as a reaction on the part of the immune organism to the subsequent resorption of spirochetic toxins. Spirochetolysis continues for the whole period of the disease, but only with the appearance of complete immunity does the organism react in the form of fever. The toxins arise from the disintegration of the spirochetes within the organs, above all, the kidneys, where probably a proliferation of spirochetes takes place during the convalescent stage, when numerous specimens can be observed. On the other hand, it must be remembered that the after fever occurs in only 28.2 per cent of the cases, while the phenomena of antibody formation and excretion of spirochetes occur with constancy. It appears that the pathogenesis of the after fever requires still further investigation.

A CONTRIBUTION TO THE ETIOLOGY AND PATHOLOGY OF RAT-BITE FEVER.*

By RENJIRO KANEKO, M.D., and KIKUZO OKUDA, M.D.

(*From the First Medical Clinic of the Imperial University in Kyushu, Fukuoka, Japan.*)

Plates 32 to 34.

(Received for publication, February 20, 1917.)

Although rat-bite fever in Japan was recognized a long time ago by Miyake and others as a definite entity, and its symptomatology has been described in detail, we know little of its pathological anatomy. According to Ishiwara only two postmortem examinations have been reported, one by Blake, and another by Miura and Toriyama.

Our knowledge of the causative agent of rat-bite fever is of recent date. Futaki and his associates reported in their first communication the finding of a long spirochete, measuring 9 to 10 μ in the lymph glands on the 10th or 13th day of illness, and on the 33rd day in tissue fluid obtained from the bitten area. Those found in the lymph exudate had small waves, those from sections of lymph glands, numerous steep waves. Later on they discovered in man, in the wound of the rat bite, and in the blood of mice which had received injections of patient's blood, shorter, thick spirochetes, 2 to 6 μ long. These had regular, close, and steep waves, and a filament at each end. As these long and short spirochetes are morphologically distinct, and both have been demonstrated in rat-bite fever, the question arose whether they are of the same species, or whether rat-bite fever may be caused by two different spirochetes.

The problem could not be solved at the time by Futaki and his coworkers. In the meantime, Ishiwara and his associates found in their investigations of experimental rat-bite fever spirochetes of the shorter variety, which were morphologically identical with the short variety of Futaki. Kitagawa and Mukoyama, as well as Midzukuchi, reported finding these spirochetes in experimental rat-bite fever.

We performed recently a postmortem examination on an individual who had rat-bite fever in typical form, and were in a position to make

* Published in *Iji-Shimbun*, June, 1916, Nos. 951, 952.

definite anatomical studies. We found in the tissues certain spirochetes which will be described, and were able to confirm the work of Futaki and his associates, who were the first to designate these spirochetes as the cause of rat-bite fever. We have furthermore come to the conclusion that the long type of spirochetes found by them previously and the short variety discovered later in experimentally infected animals are not diverse in type, but belong to the same species of spirochete.

Spirochetes in the Tissues.

We examined the blood of a patient having rat-bite fever during the afebrile interval, guinea pigs inoculated with the patient's blood, as well as the swollen lymph glands, but found no spirochetes. Later Ido and other authors found in blood films taken at the height of the disease, a number of the short type of spirochetes.

Distribution in the Organs.—We focused our attention particularly upon the suprarenals and the kidneys, in view of the fact that Ishiwara and his associates in their experiments on guinea pigs found the organisms mainly in the suprarenals, and also because *Spirochæta icterohæmorrhagiæ* is present in these organs in the convalescent stage of Weil's disease. After repeated careful examinations, we discovered numerous organisms, particularly in the kidneys.

The spirochetes in the kidneys are located almost exclusively in the casts and cylindroids of the straight tubules, in the canals of Henle, and the intercalary portion of the boundary layer. They were never found free in the lumen and the interstitial spaces. In the suprarenals, we observed the organisms in the cortical cells. In addition, we found in the interstitial space of the testicle a specimen of the short spirochete. Other organs, *i.e.*, the liver, lymph glands, spleen, lungs, intestines, stomach, bladder, prostate, heart, and brain, showed no spirochetes; nor could any be found in the skin of the bitten area, treated with Orth's fluid, and in the swollen lymph glands.

The total number of spirochetes found in the organs was somewhat over 100 in 30 preparations; most of these were contained in renal casts and cylindroids. In the suprarenals, we observed seven specimens in ten preparations, and in the testicle one in several preparations. Their distribution is very irregular; in the densest regions five to six specimens were found in one cast, and twenty-eight in a preparation.

Forms of the Spirochete.

As shown in Fig. 1, the forms of the organism are various. The spirochete may be long or short, thick or thin, rigid or pliable, smooth or ragged. The waves may be regular or irregular, gradual or abrupt, many or few. The diversity of characteristics is so great as to lead to the belief that many kinds of spirochetes are present. They can, however, be classified according to size into long and short, with intermediary forms between the two groups.

The short spirochetes found by us are from 1.7 to 5.0 μ in length. As a rule, they have close and regular waves, from two to six steep waves to a specimen, are relatively thick, with smooth edges, and stain deeply when impregnated with silver. Occasionally one finds at one or both ends, fine thread-like extensions which stain somewhat less deeply. These regularly waved spirochetes are identical with the short spirochete of Ishiwara. But a large number of the short spirochetes are more rigid in form, have irregular waves, and are not smooth at the edges. These are atrophic abortive forms. The spirochetes found in blood films have regular waves.

The long type spirochete measures 6.0 to 10.0 μ, and has numerous, small, steep, irregular waves. These specimens are not smooth and regularly formed, and appear rigid.

We examined the mouse preparations (from the injection of patient's blood) sent us by Futaki, and are convinced that his short type of spirochete is identical with our own, with the exception that our specimens were somewhat thinner and more rigid, which may be due to the fact that our strain was taken from man. Futaki's long type, which we observed in great numbers in his preparations, is morphologically identical with ours.

In addition we made careful histological examinations of guinea pigs infected by Ido and his collaborators through rat bites. The spirochetes found in these animals are morphologically identical with our short spirochete.

DISCUSSION.

In Weil's disease, the distribution of *Spirochæta icterohæmorrhagiæ* varies with the different stages of illness. At the beginning the organ-

isms circulate in the blood, while in the convalescent stage, following the complete development of immune bodies, they can be demonstrated only in the kidneys. A similar distribution seems to prevail in rat-bite fever. In the experimental animals, the spirochetes circulate in the peripheral blood. That identical conditions exist in the human body is proved by the fact that mice and guinea pigs can be infected by the inoculation of patient's blood. At the height of the disease, the short spirochete, though not numerous, has been observed microscopically in the blood. In our case we found the spirochetes located mainly in the kidneys, although at the height of illness they could be demonstrated also in the peripheral blood. The localization within the kidneys is undoubtedly the usual order in later stages of the disease.[1]

Inadà was the first to suggest the formation of antibodies in rat-bite fever. He was followed by Ido and his associates of our clinic, who proved the truth of the assumption. Judging by analogy with Weil's disease, we suggest that the irregular distribution of spirochetes in the kidneys may be attributable to the partial destruction that has taken place owing to the action of the antibodies.

The characteristics of our spirochetes—their irregularity and rigidity—may also be considered as the result of the action upon them of the immune bodies. We know that in spirochætosis icterohæmorrhagica the organisms disappear in the course of the illness through the action of the spirochetolytic and spirocheticidal antibodies, leaving various irregular so called degenerated forms of spirochete. It is highly probable that this is also the case in rat-bite fever in man. On the other hand, in the animal body, where no specific immune bodies are developed, the spirochetes exist in their typical forms as found by Futaki (short spirochete) and also by Ishiwara. In conformity with this, we have our finding of short spirochetes in the blood of patients at the height of the disease.

Finally we are confronted with the important problem as to whether the short and long spirochetes constitute two different species or one species of spirochete. Futaki seems to regard them as organisms different in type, and for that reason was not clear

[1] At the time of death the patient had passed the height of the disease.

concerning the etiology of rat-bite fever. On the basis of histological investigations, we conclude that these two forms of spirochete belong to the same species, as one type grades into the other, morphologically speaking. Moreover, it does not seem probable in our case of rat-bite fever that spirochetes of different types simultaneously infected the same individual and localized in the renal casts.

The long spirochetes occur almost exclusively in human tissues and we may regard them as old or degenerated forms which have become irregular and stiffly bent. The short spirochetes, on the other hand, which have regular waves are the typical, young specimens. They can be found in the blood of patients having rat-bite fever, as well as in the experimental animals.

Futaki and his collaborators, 2 weeks prior to the appearance of our publication, on the basis of experiments made with pure cultures, arrived at a conclusion identical with our own.

Pathological Anatomy.

Case 1.—Z. I., male, age 70, locomotive engineer. Clinical diagnosis: rat-bite fever.

History.—A month previously the patient while asleep was bitten by a rat, in the forefinger of the left hand. The wound bled. After 2 weeks the area became swollen and red; very sensitive. Incision was made. The following day the patient observed blotches, the size of a finger, in rows on the left forearm. These disappeared gradually, only four remaining. Similar spots appeared in the left cubital region and upper arm, and along the tendon of the forefinger. 20 days after the bite, the patient observed swelling of left axillary lymph glands, which became as large as a pigeon's egg, and were very painful. Temperature was not taken, but after the incision there was fever. A few days ago, slight chills, fever, headache, and loss of appetite, which have continued.

Admitted to clinic, Oct. 18, 1915.

Present Illness.—Male, medium height. Nutrition fair. Conjunctivæ, mucosa of palate, and pharynx markedly hyperemic. Tongue swollen and coated. Lymph glands in left axilla, size of bean to pigeon's egg; very sensitive. Both cubital glands swollen to size of lentil; elastic, soft, not sensitive. Inguinal glands not swollen. Thickened arterial walls. Liver palpable two fingers below costal arch, not sensitive. Spleen not palpable. No ascites. Urine negative.

Left forefinger shows scar 1 cm. at point of incision. Joint somewhat affected. Skin on dorsal side of left second metacarpophalangeal articulation somewhat eroded and scarred. Surrounding area red and hot. On dorsal sur-

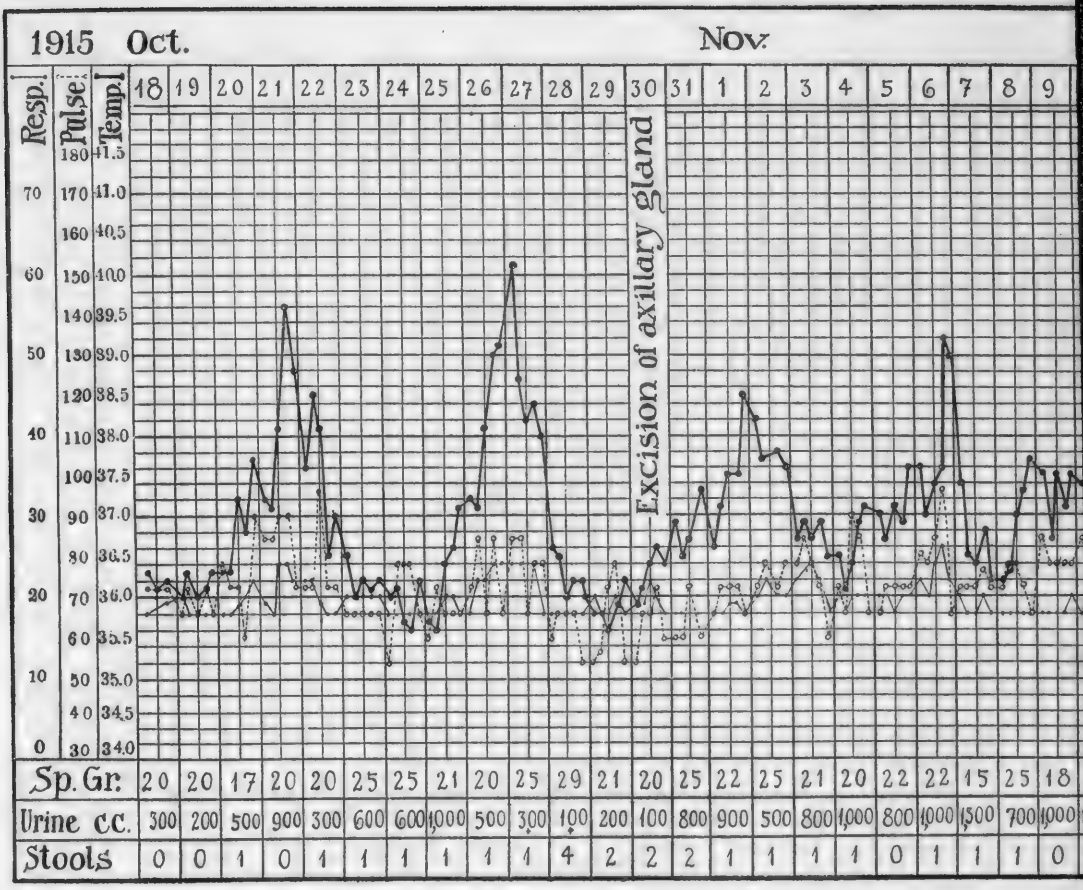
face of left hand, a number of reddened areas, size of quarter, somewhat raised, also along radial side of fore and upper arm. The spots are united by fine red stripes.

Blood.—Oct. 20, with ascending fever, hemoglobin (Sahli), 60 per cent, erythrocytes, 4,012,000, leukocytes 5,600. Oct. 29, interval; hemoglobin 64 per cent, erythrocytes 4,290,000, leukocytes 4,200. Nov. 2, rising fever, erythrocytes 3,090,000, leukocytes, 12,100.

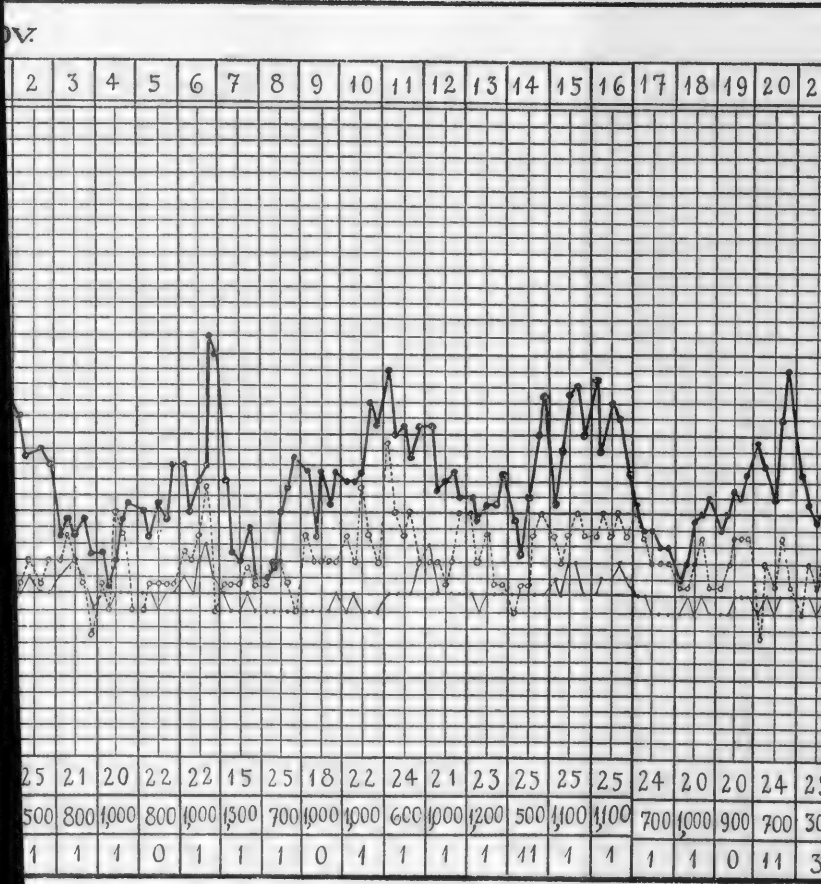
Course.—Death from senile anorexia, 7 weeks after admission. Altogether eight fever attacks, each lasting 3 to 4 days, with afebrile intervals of 2 to 3 days. At onset of illness, typical fever; later, atypical, often remittent. Height of fever in an attack 38.5–39.5°C., once reaching 40.1°C. (Text-fig. 1).

Left axillary glands at first swollen to size of pigeon's egg, diminished gradually. Oct. 30, when excised, they were the size of a bean. Gland was flat and elastic, partly hyperemic, grayish white. Spots on skin became gradually fainter; none found on Oct. 26. Gradual loss of weight. Died of inanition and marasmus, Dec. 6.

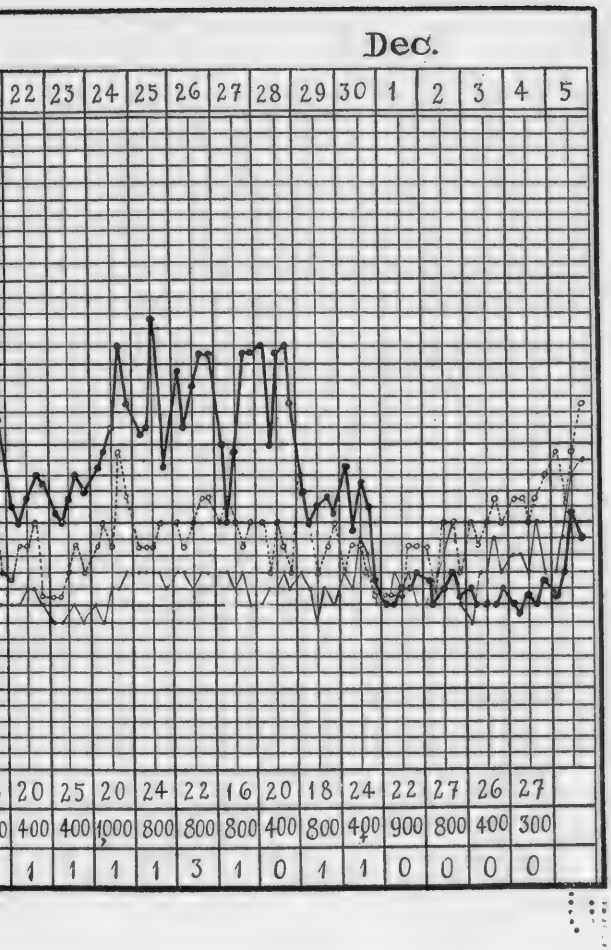
Autopsy.—9 hours later. Body poorly nourished. Rigor mortis only in knee joints. Skin dry, dark brown. Scar on left forefinger. No edema or exanthemata. Peritoneum contained no abnormal fluid. Mesenteric lymph glands swollen, on section somewhat injected. Pericardium contained a tablespoonful of serous fluid, with fibrin fragments; inner surface of pericardium opaque. Heart in diastole, size of patient's fist, weight 265 gm. In left ventricle, no fluid or coagulated blood; in right ventricle, small mass of fatty coagulum. Pericardium somewhat opaque, endocardium without luster. Intima of root of aorta, marked arteriosclerotic spots; valves negative. Myocardium soft, yellowish brown. Spleen somewhat soft, small, 8.0 by 7.0 by 1.5 cm., 64 gm., capsule corrugated, trabecular. Lungs, marked anthracosis; hyperemic; scattered atelectatic foci. Glands of hilum somewhat swollen. Kidney, left, 1.2 by 5.0 by 3.0 cm., 116 gm., capsule adherent, surface not smooth, partly nodular and granulated, congested. Large cysts scattered on surface. On section hyperemic, cortex thin; columns of Bertin yellowish, granulated; mucous membrane of pelvis slightly hyperemic, somewhat enlarged. Parenchyma friable and cloudy. Right kidney, 11.0 by 4.5 by 2.5 cm., 98 gm., similar to left. Suprarenals hyperemic. Liver, 30.0 by 13.5 by 5.5 cm., 1,062 gm. Surface smooth, rib furrow on right lobe. Parenchyma on section yellowish, dull, but acini recognizable; congested, consistency tough. Gall bladder contained greenish mucous gall; mucosa negative. Stomach contained small quantity of mucus; mucosa hyperemic and opaque. The same may be said of mucosa of duodenum; papilla duodeni permeable. Pancreas, hyperemic. Intestines, mucosa opaque and hyperemic; follicles not swollen. Bladder contained small amount of turbid urine; marked hyperemia of mucosa. Prostate slate-colored, not hypertrophied. Cranium, subdurally, large mass of serous fluid; meninges soft, somewhat opaque and hyperemic; brain slightly hyperemic. Bone marrow of tibia negative.


1915 Oct.
Nov.
Resp.
Pulse.
Temp.
18 19 20 21 22 23 24 25 26 27 28 29 30 31 1 2 3 4 5 6 7 8 9
Excision of axillary gland
Sp. Gr: 20 20 17 20 20 25 25 21 20 25 29 21 20 25 22 25 21 20 22 22 15 25 18
Urine c.c. 300 200 500 900 300 600 600 1,000 500 300 100 200 100 800 900 500 800 1,000 800 1,000 1,500 700 1,000
Stools 0 0 1 0 1 1 1 1 1 1 4 2 2 2 1 1 1 1 0 1 1 1 0

TEXT-FIG. 1.



TEXT-FIG. 1. Case 1, male.


Dec.
22 23 24 25 26 27 28 29 30 1 2 3 4 5
20 25 20 24 22 16 20 18 24 22 27 26 27
400 400 1000 800 800 800 400 800 400 900 800 400 300
1 1 1 1 3 1 0 1 1 0 0 0 0

Diagnosis.—Rat-bite fever. (1) Marasmus universalis. (2) Degeneratio parenchymatosa cordis, hepatis, renum. (3) Gastroenteritis catarrhalis. (4) Cystitis catarrhalis. (5) Edema and hyperemia meningea. (6) Arteriosclerosis gradus gravioris. (7) Nephritis interstitialis chronica.

Histological Examination. Lymph Glands.—From left axilla, removed Oct. 30, negative. Stained with hematoxylin and eosin. Showed simple hyperplasia of adenoid tissue. In capsule and its surrounding area, infiltration with plasma cells and leukocytes. Parenchyma markedly hyperemic, but showed no regressive or marked inflammatory changes.

The other organs were placed in fixing solution, and stained. The following is a report of the hematoxylin and eosin preparations.

Kidney.—By low power, hyaline degeneration of glomeruli, interstitial proliferation, and casts, as well as cylindroid formation in the tubules. Cortex and medulla hyperemic here and there. Subcapsulary lobulation of cortex, consisting of scar tissue, containing degenerated hyaline glomeruli and the remains of kidney tubules; marked cellular infiltration. By high power, marked changes in kidney tubules, particularly of the convoluted tubules. Cells markedly enlarged, partly degenerated, nuclei remaining unstained and cells necrotic, staining faintly reddish violet. Some cells when stained with Sudan show small fat globules. In other places, marked desquamation and destruction of cells, with stained nuclei. Slighter changes in wall epithelium of lower sections of convoluted tubules, Henle's loops, and the intercalary portion; nuclei stain well; no necrosis, though sometimes marked destruction and desquamation of epithelium. Infiltration of fat globules more marked in lower sections of the tubules. The epithelium of convoluted tubules contains very small fat globules resembling lipoid substance, while in the epithelium, of straight tubules, infiltration of larger fat globules is common.[2] The renal casts and cylindroids are always hyaline, and rarely contain blood cells and desquamated epithelium. Numerous urinary casts particularly in the lower sections of the tubules, and in convoluted tubules, Henle's loops, intercalary portion, etc. Now and then in the enlarged tubules one finds a round, hyaline body composed of layers. The medullary canals also contain numerous casts. The glomeruli show total or partial hyaline degeneration, some being hyperemic. No abnormal fluid in Bowman's capsules. The interstitial spaces are markedly dilated, but without new connective tissue or cellular infiltration, generally edematous, and in part transformed into hyaline. The blood vessels show no marked changes.

Liver.—Cells stain faint reddish violet with hematoxylin and eosin, often contain fat globules, fine vacuoles, and yellowish brown pigment bodies in the neighborhood of the nuclei. No marked swelling or opacity of liver cells. With Sudan III, the cells in acini centers show infiltration with small fat globules, while

[2] This phenomenon was also observed by us in spirochætosis icterohæmorrhagica.

in the cells of the acini periphery there is an accumulation of large drops of fat. The central parts of the acini are broken up and markedly degenerated. The cells are atrophic, disintegrated, or necrotic; many have disappeared. The gaps are filled with blood cells; various grades of necrobiosis in cells. Nuclei are unstained or disintegrated; some have disappeared. Marked fatty degeneration. Liver cells of acini periphery are relatively compact. Stellate cells of Kupffer slightly enlarged; in part fatty degeneration has taken place. Some contain blood cells and pigment granules. The interstices show no marked changes. Slight infiltration of round cells here and there. In the bile duct nothing noteworthy. Wall epithelium rarely shows infiltration of fat globules.

Spleen.—Negative. Follicles not enlarged, trabeculæ well developed. Scattered hyperemia. Central arteries thickened, intima shows hyaline degeneration. The nuclei of scattered pulp cells do not stain well; destroyed. No large necrotic foci, cellular infiltration, pigment deposits, erythrophagocytosis, or microorganisms.

Suprarenals.—Show in deep layer of cortex circumscribed hyperemia, causing slight scattering of cells.

Cardiac Muscles.—Somewhat pale and indistinct; no fatty or other degenerations present. In the fibers near the two ends of the nuclei, numerous pigment bodies, which stain yellowish with Sudan III (brown atrophy).

Lungs.—Catarrhal changes in consolidated parts.

Mesenteric Glands.—Blood vessels in the medulla are markedly dilated and filled; lymph sinuses dilated. Endothelium, desquamated and degenerated, often taking up erythrocytes. Some scattered degenerated cells in sinus and follicles. Mucosa of gastrointestinal tract covered with mucus; epithelium desquamated, and cellular infiltration in propria.

Muscle fibers of gastrocnemius here and there slightly swollen; partly changed into hyaline. No marked destruction of fibers or fatty degeneration.

Nerve Cells.—Cells of brain cortex and spinal cord here and there are slightly enlarged or destroyed; some are pyknotic, nuclei not stained. Nerve fibers show, though rarely, slight swelling and disintegration.

Skin.—Shows scar at site of bite.

Case 2.—R. H., female, age 40, peasant woman. Admitted to Second Medical Clinic, July 28, 1908.

History.—70 days ago, while asleep, the patient was bitten in the upper lip by a rat. The wound bled, but healed in a few days. After a month, the skin of the bitten area became red, swollen, and very sensitive. Swelling and redness extended gradually to the angle of the mouth on the right side, and then to the submaxillary region. Since that time, patient had attacks of chills and fever, with headache and malaise. First attack lasted for a week, followed after an interval of 1 week by a second. Patient observed also about ten scattered spots, varying from the size of a finger point to a walnut, on the extremities and the chest. High fever with nausea, vomiting, loss of appetite, headache, etc.

Swelling of lip and redness subsided gradually. Fever came in attacks, often accompanied by vomiting.

Present Illness.—Medium height and nutrition. At left angle of mouth, a reddish brown spot, 2 cm. in width. Neck in front shows numerous spots, some connected. Extremities also show spots, 1 to 4 cm. in width. Over the entire body, scattered exanthematous areas, which disappear on pressure. Tongue coated. Pulse 120, regular, tense. Breasts and abdominal organs negative. Biceps and brachialis anticus somewhat sensitive. No albumin in urine. No edema. Temperature 38.8°C.

Course.—July 30. Blood: leukocytes 14,333, erythrocytes 3,495,000. Aug. 8. Rising fever, up to 38.7°C. Vomiting. Aug. 10. Muscular pains in lower extremities. Aug. 12. Disappearance of spots and redness of mouth region. Aug. 15. Vomiting. Aug. 18. Rapid pulse. Aug. 20. Fever, 39°C., increased pulse (150 per minute), regular, small. No urination, marked meteorism. Aug. 22, unconscious. Death by cardiac insufficiency.

Autopsy.—12 hours after death. Height 150 cm., weight 36.4 kg. Nutrition poor. Rigor mortis only in knee joints. Diffuse postmortem discoloration on back and neck. Abdomen somewhat distended. Skin pale and dry, no exanthemata, no edema. In abdominal cavity about 100 cc. of clear yellowish fluid. Intestines moderately distended with gas. Serous membranes of intestines pale, slate-colored. Diaphragm, height of fourth intercostal space. No abnormal fluid in thorax. Lungs, no adhesions. Pericardium contained about a tablespoonful of clear yellow fluid; inner surface of pericardium somewhat injected. Heart, size of patient's fist. In right ventricle a large amount and in left ventricle moderate amount of dark red fluid blood and fatty coagulum. Epicardial fat tissue moderate, somewhat edematous. Endocardium smooth, myocardium somewhat opaque. Consistency of heart soft. Intima of root of aorta shows in the region of the aortic valves a number of yellow plaques. Left lung voluminous. Lower edge of lower lobe shows hypostatic induration. On section, congested. Bronchial mucosa markedly hyperemic. Peribronchial glands partly swollen and markedly anthrocotic. Right lung moist and congested, resembling left. Spleen, 12.0 by 5.0 by 2.0 cm., weight 58 gm. Capsule wrinkled. On section congested, follicles and trabeculæ visible, consistency soft. Left kidney, 11.5 by 6.0 by 2.5 cm., 117 gm. Capsule easily removed. On outer surface, urinary cyst the size of a pea. Parenchyma shows slight opacity. Columns of Bertin somewhat swollen, parenchyma yellowish, boundary of both substances partly indistinct. Right kidney, 11.3 by 6.0 by 2.8 cm., 121 gm., resembling the left. Liver, 24.0 by 16.0 by 6.0 cm., 1,340 gm. Surface smooth. On section somewhat yellowish and opaque. Gall bladder contained dark green gall. Mucosa negative. Stomach contained a large mass of sour smelling grayish green food. Mucosa in spots hyperemic. Intestines, small, held a large mass of dark yellowish green contents. Mucosa slate-colored or greenish yellow, somewhat edematous; scattered small nodules the size of pin-head.

These nodules are dark brown, somewhat coarse, and yellowish in the center. Lower section of small intestine contained twenty-four specimens of *Ascaris lumbricoides;* mucosa markedly injected. Large intestine contained small fecal (fluid) mass; mucosa somewhat injected. Retroperitoneal and mesenteric lymph glands not markedly swollen. Urinary bladder contained small amount of turbid urine; mucosa opaque and injected. Mucosa of vagina also somewhat injected. Portio uteri red. Mucosa of uterus slightly hyperemic.

Diagnosis.—Rat-bite fever. (1) Marasmus universalis. (2) Degeneratio parenchymatosa cordis, hepatis, renum. (3) Gastroenteritis catarrhalis. (4) Cystitis catarrhalis. (5) Hydrocele tubæ dextræ. (6) Ascaridosis.

No microscopic examination.

Case 3.—N. H., female. Examination of swollen axillary gland excised at beginning of illness showed marked hyperplasia of parenchyma cells. The follicular substance is particularly hyperplastic. In places, the hemorrhages in the follicles are marked, leaving small islands of cells between. No necrosis of the parenchyma. In the peripheral zone of the gland there is quite a dense accumulation of polynuclear leukocytes. Corresponding with the site of hemorrhage, the epithelium shows here and there desquamation and degeneration. Erythrophagocytosis is present, but not as plainly as in spirochætosis icterohæmorrhagica. In the medullary substance, scattered leukocytes are found.

Case 4.—Skin preparation (old) from area bitten by rat. Corium and subcutaneous tissue markedly hyperemic, edematous, and infiltrated with polynuclear leukocytes. No abscess formation. Corium tissue shows hemorrhages and fibrin deposits. No changes in epidermis. Scattered necrotic cells in infiltration zone, but no extensive necrotic foci.

SUMMARY AND DISCUSSION.

Of the two postmortem examinations on rat-bite fever previously reported, the patient of Miura and Toriyama, a woman, aged 32 years, died on the 70th day after the onset of the disease. Anatomically there were no marked changes. Histological examinations were not made. Macroscopically, the following findings are reported: increase of cerebrospinal fluid, hyperemia of the meninges, inflammatory edema of the lungs, cloudy swelling of the liver, etc.

The other, Blake's case, was a woman of 67, who died 1 month after the bite of a rat. The anatomical findings were acute ulcerative endocarditis, subacute myocarditis, interstitial hepatitis, glomerular and interstitial nephritis. The suprarenals contained a large amount of perivascular fluid. There were infarcts in the spleen and kidney. In general, the organs were hyperemic; the lung showed hemorrhages and edema. Histologically, Blake found marked degeneration, necrosis, and infiltration in the cardiac muscles, infiltration of polynuclear cells, and increase in the interstices of the liver, also marked polynuclear infiltration of the shrunken kidney.

Blake was able to demonstrate *Streptothrix muris ratti* in the tissues, which he assigned as the cause of rat-bite fever. He viewed the degeneration and infiltration in the organs, together with the ulcerative endocarditis, as the result of the streptothrix infection.

Pathological Anatomy.

Our Case 1 was that of a man of 70, who died 80 days after the bite of a rat, and 2 months from the onset of illness. In contrast to Blake's case, there were no marked pathological changes, with the exception of the parenchymatous changes in the organs. The kidney changes are attributable in part to the condition of shrunken kidney, which had existed for some years previous to the infection. Other changes, such as the marked hyperemia, swelling, and degeneration of the tubular epithelium, must be regarded as caused by the rat-bite fever. In part, the formation of casts can likewise be so interpreted. The liver shows marked changes, degeneration, necrosis, and destruction of liver cells, particularly in the acinus centers, with hyperemia and hemorrhages, the fatty degeneration of the liver cells being especially marked in the acinus centers. Parenchymatous changes of the liver and kidney were observed macroscopically in Case 2.

The hematopoietic organs, *i.e.*, the spleen, lymph glands, and the bone marrow, show in general no abnormalities. The local lymph glands at the beginning of illness show marked hyperplasia of the parenchyma cells. In this, the part played by the follicular substance must be taken into consideration. The cellular hyperplasia is simpler and of an inflammatory character. Although the hyperemia, hemorrhages, and the loosening of the follicles are distinct, there are no noteworthy regressive changes. There is a slight catarrhal condition of the sinus and erythrophagocytosis, while in spirochætosis icterohæmorrhagica these manifestations are marked. In the course of the disease, the swelling of the local lymph gland subsides gradually, and the corresponding changes become less. Other lymph glands, such as the mesenteric, also show slight swelling, with hyperemia and catarrhal changes.

In addition we noted in our case catarrhal changes in the mucous membrane of the stomach, cystitis catarrhalis, congestion of the

lungs, etc. The degeneration of the muscle and nerve cells, though slight, must be regarded as a phenomenon of this disease. The meninges were also hyperemic and edematous.

We have few comments to make on the exanthemata, as we lacked material for anatomical investigation. The site of the bite showed, as in the fourth case, cellular infiltration, edema, and degeneration (acute exudative inflammation). In the lungs, as already mentioned, we found signs of congestion, but the accompanying marked inflammatory changes must be regarded as the result of a mixed infection, because we found an accumulation of diplococci.

The changes observed by Miura and Toriyama agree with ours, but a great difference exists between Blake's case and ours. In his case there were marked infiltration of pus cells and regressive changes in the organs—even an ulcerative endocarditis. These differences cannot be explained on the basis of the duration of illness. Though the time of illness in our case was very long; it was marked up to the time of death by fever. In Blake's patient, we must attribute the marked changes, as emphasized also by the author, to streptothrix infection. If this streptothrix is the cause of rat-bite fever, the changes which have been described would be significant, but in view of our findings in Japan, and on the basis of the histological studies made by Ido, Ito, Wani, and Okuda,[3] there is doubt in our minds whether the streptothrix should be so regarded. It is possible that Blake's case was one of mixed infection.

In their experiments with rat-bite fever, Ishiwara and his associates found in addition to swelling of the bitten area and lymph glands, acute inflammatory changes in the kidney, and hyperemia and hemorrhages in the suprarenals. We examined the guinea pigs of Ido and his associates, which were infected by the bite of rats; and also observed swelling of the bitten area and of the regional lymph glands, with slight nephritis, hyperemia of the lungs, catarrh of the mucosa of the stomach, and slight parenchymatous degeneration in the organs. These changes on the whole resemble those of human cases.

[3] Ido, Y., Ito, H., Wani, H., and Okuda, K., Circulating immunity principles in rat-bite fever, *J. Exp. Med.*, 1917, xxvi, 377.

In conclusion we desire to express to Dr. Ryokichi Inada our thanks for his assistance in this work, to Dr. H. Nakayama of the Pathological Institute of the Imperial University in Kyushu for permitting us to use the protocols, and to Dr. Takeya of the Second Medical Clinic of the University for the use of patients' histories.

BIBLIOGRAPHY.

Blake, F. G., *J. Exp. Med.*, 1916, xxiii, 39.
Futaki, Takaki, Taniguchi, and Osumi, *Tokyo Igaku-Kwai-Zasshi*, 1915, xxix, No. 23.
Futaki, Takaki, Taniguchi, and Osumi, *Igaku Chuozasshi*, 1916, No. 230, 1481.
Futaki, Takaki, Taniguchi, and Osumi, *J. Exp. Med.*, 1916, xxiii, 249.
Futaki, Takaki, Taniguchi, and Osumi, *J. Exp. Med.*, 1917, xxv, 33.
Ishiwara, Ohtawara, and Tamura, *Nippon Eisei Gaku-Kwai Zasshi*, 1916, xi, No. 6.
Ishiwara, K., Ohtawara, T., and Tamura, K., *J. Exp. Med.*, 1917, xxv, 45.
Kitagawa and Mukoyama, *Igaku Chuozasshi*, 1916, No. 230, 1483.
Midzukuchi, *Igaku Chuozasshi*, 1916, No. 230, 1485.
Miura and Toriyama, *Tokyo Igaku-Kwai-Zasshi*, 1897, xi, No. 23.

EXPLANATION OF PLATES.

PLATE 32.

FIG. 1. Various forms of spirochetes found in the tissues. From silver preparations.

PLATE 33.

FIG. 2. Spirochetes found in renal casts. From silver preparations.

PLATE 34.

Spirochetes in the tissues.

FIG. 3. Spirochete in cortex of suprarenal. × 1,130.

FIGS. 4 to 6. Spirochetes in urinary casts. Fig. 4, × 630. Fig. 5, × 380. Fig. 6, × 460.

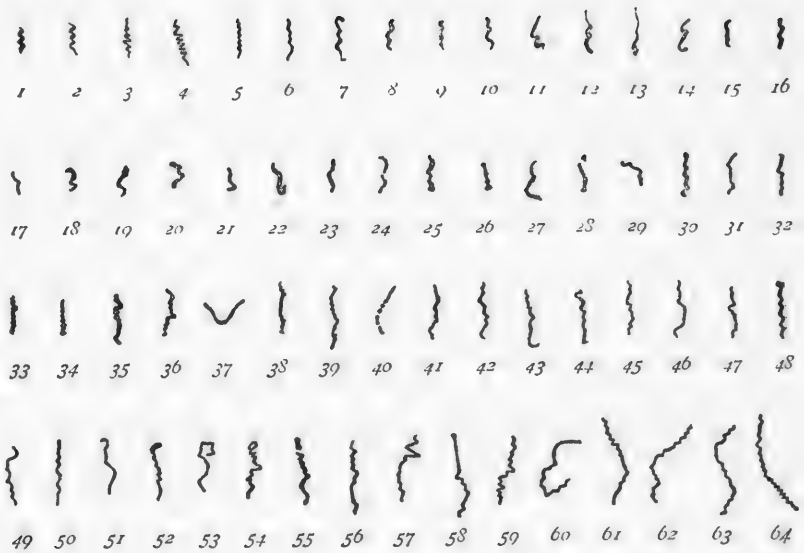

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32
33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48
49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64

FIG. 1.

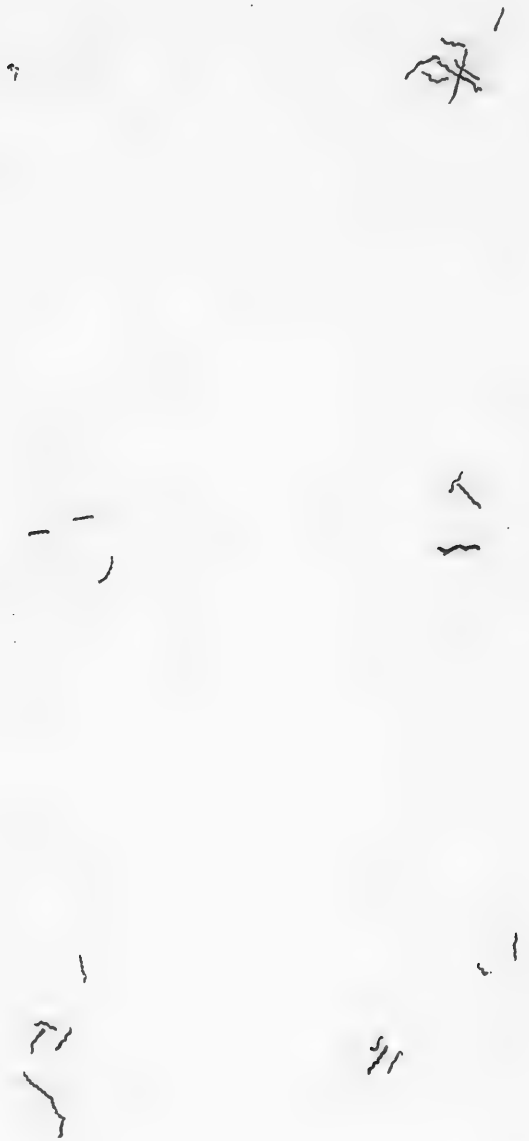


FIG. 2.

(Kaneko and Okuda: Rat-bite fever.)

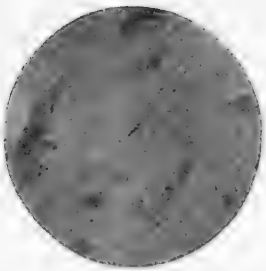


FIG. 3.

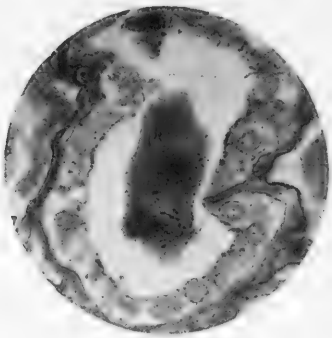


FIG. 4.

FIG. 5.

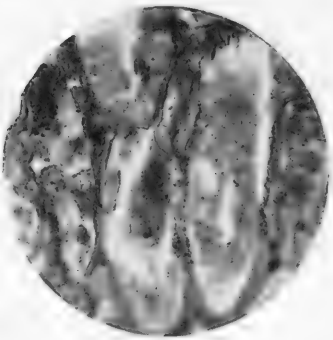


FIG. 6.

(Kaneko and Okuda: Rat-bite fever.)

CIRCULATING IMMUNITY PRINCIPLES IN RAT-BITE FEVER.*

By YUTAKA IDO, M.D., HIROSHI ITO, M.D., HIDETSUNE WANI, M.D., AND KIKUZO OKUDA, M.D.

(*From the First Medical Clinic of the Imperial University in Kyushu, Fukuoka, Japan.*)

(Received for publication, February 20, 1917.)

We owe our knowledge of the causative agent of rat-bite fever largely to the researches of the Japanese workers, Futaki and Ishiwara, and their associates. Futaki in 1915, reported finding a spirochete in preparations from the bitten area and the lymph glands of persons having rat-bite fever. Ishiwara and Ohtawara, during the same year, conducted histological studies on guinea pigs which had been experimentally infected with rat-bite fever, and found a similar spirochete. In 1916, numerous other authors in Japan took up the problem which centered particularly in the variety of types of spirochetes found in the disease in man and the experimental animals. Two main groups—long and short spirochetes—were distinguished. In May, 1916, Futaki announced on the strength of cultural studies made by him and his associates, that the two types are in all probability identical, and a little later he definitely advanced the belief that the organism is the causative agent of rat-bite fever. His work was confirmed by Kaneko and Okuda,[1] who reported on the pathological anatomy of the disease.

The short spirochete found by Futaki, and Kaneko and Okuda, coincides with respect to form and movement with that observed by Ishiwara and his associates in experimentally infected guinea pigs. These spirochetes have been identified in guinea pigs, white rats, and mice, following the intraperitoneal injection of patients' blood and emulsion of lymph gland. In the guinea pig, fever resulted. In no instance were these spirochetes found in healthy guinea pigs, white rats, or mice. They were also absent from the blood and tissues of infected animals receiving salvarsan treatment.

On the basis of the facts above cited we may say that in all probability the spirochete which has been described is the causative agent of rat-bite fever. The premises are, however, not complete, for we

* Published in *Tokyo Iji-Shuho*, September, 1916, No. 1990.

[1] Kaneko, R., and Okuda, K., A contribution to the etiology and pathology of rat-bite fever, *J. Exp. Med.*, 1917, xxvi, 363.

lack one significant proof in order to make the assertion conclusive. It is necessary to demonstrate in the serum of patients having rat-bite fever an immunity principle which is specific for the spirochetes of that disease. Up to the present time such confirmation has been lacking.

In the postmortem examination made in our clinic of a patient having rat-bite fever, the spirochetes were found located mainly in the kidneys, and not in other tissues. A similar localization of spirochetes in the kidneys is true in the convalescent stage of spirochætosis icterohæmorrhagica, and as that phenomenon, together with the disappearance of symptoms in Weil's disease, has been explained on the ground of the formation of antibodies in the blood, we may assume that a similar condition exists in rat-bite fever; *i.e.*, antibodies are present in the blood of those who recover from the disease. In order to prove this point, we obtained blood serum from three patients recovering from rat-bite fever, and conducted the experiments cited below.

The spirochetes employed by us came from guinea pigs, the animals having been infected through the bite of the rat, *Mus decumanus*. Ishiwara has affirmed that these organisms are morphologically identical with those studied by him and obtained from experimentally infected animals. We used no spirochetes taken directly from human beings.

Histories of Patients from Whom Blood Was Obtained for Animal Experiments.

Case 1.—Male, age 17, farmer. In Aug., 1915, the patient was bitten in the tip of the right little finger by a house rat. After 2 weeks, the bitten area became swollen and red, followed by swelling of right neck and axillary glands, and typical fever. Admitted to First Surgical Clinic of the University. Diagnosis, rat-bite fever. Discharged, cured, after 59 days. Blood taken on July 27, 1916.

Case 2.—Male, age 40, farmer. The patient was bitten in the upper arm by a rat, in Feb., 1916. After 20 days, the patient developed high fever, chills, swelling and sensitiveness of right axillary glands; typical exanthematous spots. Admitted to First Surgical Clinic. Treated with salvarsan. Blood taken on Aug. 4, 1916.

Case 3.—Female, age 35, wife of merchant. In May, 1916, the patient was bitten on the right upper eyelid, by a house rat. After 3 weeks, swelling and redness of bitten area; eye closed. High fever, swelling of neck glands, and exanthematous spots. Admitted to the Dermatological Clinic. Diagnosis, rat-bite fever. Blood taken on Aug. 7, 1916.

Dark-Field Preparations.

We examined by dark-field illumination a mixture of one loopful of guinea pig blood containing one to two spirochetes to an optical field and one loopful of serum from recovered cases of rat-bite fever. Control experiments were made with serum obtained from recovered cases of Weil's disease or beri-beri, and isotonic salt solution.

In the control experiments we found relatively numerous spirochetes, but we were unable to identify any actively moving organisms in the experiments made with the spirochetes and serum of rat-bite fever, although motionless or very inactive specimens were present. Here the spirochetes had been almost totally destroyed. We found only one or two specimens in a preparation.

Experiments were then undertaken to ascertain the efficacy of diluted serum. For this purpose we employed serum in isotonic salt solution, diluted two-, four-, and eightfold, and made tests similar to those which have been described. Twice diluted serum still showed the spirocheticidal and spirochetolytic properties, but with serum in a dilution of four, no such effects were observed.

It is evident from these experiments that the serum of patients convalescing from rat-bite fever contains a specific immunity principle against the spirochetes of that disease, although the immune body is relatively weak.

Pfeiffer's Phenomenon.

Pfeiffer's tests were made with the rat-bite fever serum and the blood of guinea pigs containing the spirochetes. In view of the fact that the immune bodies which develop in the blood in rat-bite fever are relatively weak, as demonstrated by the experiments described above, we employed in these tests two parts of serum to one part of

blood, in order to obtain conclusive results. The quantities used were 2 cc. of serum and 1 cc. of heart blood taken from an experimentally infected guinea pig. This mixture was injected into the peritoneal cavity of another guinea pig. After 30 minutes and again after 2 hours peritoneal fluid was drawn by puncture, and a search for spirochetes was made by dark-field illumination.

The experimental animals were also kept under observation, in order to throw further light on our results, and two series of control experiments were carried out. In the one case, we made Pfeiffer's tests with the serum of beri-beri or spirochætosis icterohæmorrhagica; in the other, we injected 1 cc. of the infected guinea pig blood but no serum. The results are shown in Tables I, II, and III.

It will be seen that no spirochetes were found in the peritoneal fluid taken 30 minutes or 2 hours after injection in Pfeiffer's tests made with the immune serum of rat-bite fever and the blood of guinea pigs containing the rat-bite fever spirochetes. On the other hand, the peritoneal fluid contained numerous, briskly moving spirochetes in the control experiments made with beri-beri or spirochætosis icterohæmorrhagica serum, as well as in the experiments where only 1 cc. of guinea pig blood, and no serum, was injected. It is clear that the serum of persons recovering from rat-bite fever has a specific spirochetolytic and spirocheticidal effect upon the organisms in question.

The blood of the experimental animals kept under observation was examined by dark-field illumination; in the case of the animals injected from Case 1 on the 12th, 20th, and 29th day after injection; of animals receiving blood from Case 2, on the 7th, 15th, and 24th day; and animals infected from Case 3, on the 5th, 13th, and 22nd day. The results in these experiments were similar to those of Pfeiffer's phenomenon tests. In the case of the guinea pig showing positive spirochetolysis, no spirochetes could be detected in the peripheral blood, while in the control animals numerous organisms were found 5 days after injection, thus proving the efficacy of the serum in protecting the guinea pig against rat-bite fever infection.

TABLE I.

Case 1.

Guinea pig No.	Injections into peritoneal cavity.		Spirochetes in peritoneal fluid.		Further course of guinea pigs. Spirochetes found in blood.		
	Patient furnishing serum. Quantity.	No. of guinea pig furnishing blood. Quantity.	After 30 min.	After 2 hrs.	12th day.	20th day.	29th day.
1	H. 2 cc. rat-bite serum, filtered.	1 cc. blood, R 27/16 V (1 spirochete in one field).	0 in one preparation.	0 in one preparation.	0 in one preparation.	0 in one preparation.	0 in one preparation.
2	" " "	" " "	0 " " "	0 " " "	0 " " "	0 " " "	0 " " "
3	" " "	" " "	0 " " "	0 " " "	0 " " "	0 " " "	0 " " "
4	" " "	1 cc. blood, R 30/16 V (1 spirochete in two fields).	0 " " "	0 " " "	0 " " "	0 " " "	0 " " "
5	" " "	" " "	0 " " "	0 " " "	0 " " "	0 " " "	0 " " "
	Control experiments.						
6	R. 2 cc. beri-beri serum.	1 cc. blood, R 32/16 V (1 spirochete in two fields).	1 in one specimen.*	1 in one specimen.	5–6 in one specimen.	2–3 in one specimen.	5–6 in one field.†
7	" " "	" " "	2–3 " " "	1–2 " " "	2–3 " " "	1–2 " " "	3–4 " " "
8	" " "	" " "	2–3 " " "	1–2 " " "	Died on 4th day, diarrhea.		
9		" " "	1 " " "	1 " " "	Killed on 4th day, diarrhea. Spirochete found in blood.		

* Cover-glass preparation, 70 optical fields, Leitz oc. 3, obj. $\frac{1}{12}$ oil immersion.

† Optical field, Leitz oc. 3, obj. $\frac{1}{12}$ oil immersion.

TABLE II.

Case 2.

Guinea pig No.	Injections into peritoneal cavity.		Spirochetes in peritoneal fluid.		Further course of guinea pigs. Spirochetes found in blood.		
	Patient furnishing serum. Quantity.	No. of guinea pig furnishing blood. Quantity.	After 30 min.	After 2 hrs.	7th day.	15th day.	24th day.
10	E. 2 cc. rat-bite serum, filtered.	1 cc. blood, R 31/16 V (more than 1 spirochete in field).	0 in one preparation.	0 in one preparation.	0 in one preparation.	0 in one preparation.	0 in one preparation.
11	" " "	" " "	0 " " "	0 " " "	0 " " "	0 " " "	0 " " "
12	" " "	" " "	0 " " "	0 " " "	0 " " "	0 " " "	0 " " "
	Control experiments.						
13	K. 2 cc. beri-beri serum.	1 cc. blood, R 34/16 V (more than 1 spirochete in field).	4 in one specimen.*	3–4 in one specimen.	1–2 in one specimen.	1 in three to four fields.†	2 in one field.
14	" " "	" " "	1–2 " " "	2–4 " " "	1 in two to three specimens.	1 in one field.	4–5 " " "
15	" " "	" " "	4 " " "	4–5 " " "	1–2 in one specimen.	1 in one to two fields.	1 in one to two fields.
16		" " "	5–6 " " "	4 " " "	1–2 " " "	1–2 in one field.	3–4 in one field.

* Cover-glass preparation, 70 optical fields, Leitz oc. 3, obj. $\frac{1}{12}$ oil immersion.

† Optical field, Leitz oc. 3, obj. $\frac{1}{12}$ oil immersion.

TABLE III.

Case 3.

Guinea pig No.	Injections into peritoneal cavity.		Spirochetes in peritoneal fluid.		Further course of guinea pigs. Spirochetes found in blood.		
	Patients furnishing serum. Quantity.	No. of guinea pig furnishing blood. Quantity.	After 30 min.	After 2 hrs.	5th day.	13th day.	22nd day.
17	G. 2 cc. rat-bite serum, filtered.	1 cc. blood, R 29/16 V (1–2 in one field).	0 in one preparation.	0 in one preparation.	0 in one preparation.	0 in one preparation.	0 in one preparation.
18	" " "	" " "	0 " " "	0 " " "	0 " " "	0 " " "	0 " " "
19	" " "	" " "	0 " " "	0 " " "	0 " " "	0 " " "	0 " " "
	Control experiments.						
20	H. 2 cc. spirochætosis icterohæmorrhagica serum, filtered.	1 cc. blood, R 30/16 V (2 in one field).	5–6 in one specimen.*	6 in one specimen.	1 in two to three specimens.	5–6 in one specimen.	3–4 in one field.†
21	" " "	" " "	7 " " "	5 " " "	" " "	2–3 in one field.	2–3 " " "
22	" " "	" " "	7–8 " " "	7 " " "	" " "	Died.	
23		1 cc. blood, R 29/16 V (1–2 in one field).	5 " " "	6 " " "	" " "	7–8 in one specimen.	1–2 in one field.

* Cover-glass preparation, 70 optical fields, Leitz oc. 3, obj. $\frac{1}{12}$ oil immersion.

† Optical field, Leitz oc. 3, obj. $\frac{1}{12}$ oil immersion.

The Action of Rat-Bite Fever Serum on the Spirochetes Circulating in the Blood.

We injected 1 to 3 cc. of serum from the three cases of rat-bite fever intraperitoneally, intravenously, or subcutaneously into guinea pigs. The results were wholly negative, as the spirochetes in the circulating blood were not affected. We believe, however, that the negative character of the experiments is due to the small quantity of serum employed, and that in order to obtain a decisive result it is necessary to use for injection into the experimental animals serum in quantities corresponding to the total amount of blood employed.

As the guinea pig requires a large amount of serum on account of its size, we used mice in our further experiments. The maximum body weight of a mouse is 10 gm., and hence the amount of serum needed is not large; we found that 1 cc. of serum is effective in the mouse. This quantity of the immune serum of rat-bite fever was injected intraperitoneally into two mice, and intravenously into another. In the mouse receiving the intravenous injection, we were unable to find, 30 minutes after inoculation, any spirochetes in the blood by dark-field illumination, while numerous organisms had been detected prior to the injection. In the other mice, those which had received intraperitoneal injections, the number of spirochetes 30 minutes after inoculation was greatly decreased, only one or two specimens being found in a preparation after diligent search. It must be added, however, that in the latter case an increase in the number of spirochetes in the blood took place 1 or 2 days later. In the intravenously injected mouse, a small number of spirochetes could be detected in the blood on the 3rd day following the injection.

SUMMARY.

Summarizing the results that have been cited, we have proved that the blood serum of convalescents from rat-bite fever contains antibodies which are specific against the causative agent of that disease. The serum of rat-bite fever was capable of destroying the spirochetes not only in the hanging drop preparations, but also in the peritoneal cavity of guinea pigs. The guinea pigs employed for

Pfeiffer's test always remained well. In the experimentally infected mice receiving intravenously or intraperitoneally serum equalling in quantity the amount of infected blood, the numbers of spirochetes were greatly decreased or they disappeared for a definite period. It is not yet clear how long after recovery from rat-bite fever the antibodies are effective in the blood of human beings, and further investigations are needed to elucidate this point. In our experiments we found that serum taken from Case 1 showed definite spirochetolytic and spirocheticidal properties 11 months after the onset of the disease. In Case 2, the period was 6 months, and in Case 3, 3 months following the onset of rat-bite fever.

CONCLUSIONS.

1. The serum of persons who have recovered from rat-bite fever contains an immune body which destroys the spirochetes of that disease.

2. The experiments here cited confirm the findings of Futaki and his associates, with respect to the spirochete which is identical with the organism discovered by Ishiwara and Ohtawara and advanced as the causative agent of rat-bite fever. The etiology of rat-bite fever has, therefore, been definitely established.

3. The immune body present in blood serum during the convalescent stage of rat-bite fever is not equal in efficacy to that contained in the serum of spirochætosis icterohæmorrhagica.

We take pleasure in expressing our appreciation of the direction and support which Dr. Ryokichi Inada has given to our work.

PULMONARY EMBOLISM.

An Experimental Study.

By FRANK C. MANN, M.D.

(From the Mayo Clinic, Rochester, Minn.)

Plates 35 and 36.

(Received for publication, April 4, 1917.)

Virchow[1] was the first to recognize embolism and to study it experimentally. Cohnheim[2] was the next investigator to consider the subject. Cohn[3] produced a classical work on embolism in which he considered the subject in its broad aspects both clinically and experimentally. Welch[4] presented the more recent complete review of the subject, and the clinical and pathologic aspects of pulmonary embolism have been discussed by Wilson,[5] who suggested this investigation.

Many experiments by various investigators have been performed on embolism. However, the main purpose of most of the experimenters has been either to determine the location of the emboli or to study the method by which the lung becomes infarcted. Very few have aimed to determine the mechanism producing death.

Deaths due to pulmonary embolism may be divided into three groups:[6] (1) Immediate death occurring when only a small portion of the pulmonary circulation is obstructed. (2) Death caused within a few minutes and due to a complete or almost complete blocking of

[1] Virchow, R. L. K., Ueber die Verstopfung der Lungenarterie, *Notizen Gebiete Natur.-u. Heilk.*, 1846, xxxvii, 26–31.

[2] Cohnheim, J., Untersuchungen über die embolischen Processe, Berlin, 1872.

[3] Cohn, B., Klinik der embolischen Gefässkrankheiten, mit besonderer Rücksicht auf die ärztliche Praxis, Berlin, 1860.

[4] Welch, W. H., Thrombosis and embolism, in Allbutt's System of medicine, London, 1899, vi, 155–285.

[5] Wilson, L. B., Fatal post-operative embolism, *Ann. Surg.*, 1912, lvi, 809–817.

[6] Schumacher, Quoted by Meyer, W., The surgery of the pulmonary artery, *Tr. Am. Surg. Assn.*, 1913, xxxi, 223–248.

the pulmonary circulation. (3) Delayed death, the result of an increase by thrombosis of an initial blockage by an embolus of a portion of the pulmonary circulation.

The cause of death in either Group 2 or Group 3 is very evident. The mechanism by which death is produced by an embolus which blocks only a small part of the pulmonary circulation (Group 1) is unknown.

The present investigation was made for the purpose of determining this unknown factor, a purpose I have not been able to accomplish as it has been possible to produce death experimentally only by a more or less complete blocking of the pulmonary circulation. However, a brief report of the experiments may be of value.

All experiments, unless otherwise stated, were performed under ether anesthesia, and the carotid blood pressure and respiration were recorded. The emboli were sent into the venous circulation through the right femoral vein except in a few experiments in which the left external jugular was used.

The emboli employed were of two kinds. One kind was made of paraffin with a melting point of about 43°C. It was found that ordinary Christmas candles offered ideal material for these. By using the different colors it was possible to make each embolus distinctive and thus to tell definitely the relationship between the time the embolus was sent into the circulation and the position in which it was found at autopsy. Furthermore, the melting point of the candle was such that it became soft and would readily mold at body temperature but did not form droplets.

The other kind of embolus was made from the animal's own blood. The left external jugular vein and the right femoral vein were dissected free for a portion of their course. Blood vessel clamps were placed on them and the exposed portion of the veins was allowed to become distended with blood. It was then gently crushed with a hemostat, and after this a few cubic centimeters of tissue extract or blood serum from the same animal were injected into the damaged veins. Under these conditions large clots formed in the vessels very quickly. When the clamps were removed the clots were swept into the circulation, the process simulating the detachment of a thrombus in a patient.

The general results of all the experiments were the same. It was impossible to produce death or seriously imperil the life of the animal by emboli until the pulmonary circulation was greatly obstructed (Protocol 1). Some emboli passed from the femoral vein to a branch of the pulmonary artery without producing any effects on either blood pressure or heart beat. Usually, however, there was a slight drop in the blood pressure at the instant the embolus passed through the heart. This drop simulated that of a momentary inhibition of the heart. Section of the vagi, however, did not prevent it. In all probability it was due to a passage of the embolus through the pulmonary valves. This was quickly recovered from and the blood pressure usually maintained a practically uniform level until many emboli had been sent into the circulation. The first noticeable effect of the emboli was an increase in the venous pressure. The abdominal veins stood out prominently, and small veins severed in the operative procedure which did not bleed at the time of section began to bleed after the passing of a few emboli. Later, blood pressure decreased; in some experiments suddenly, in others it fell to zero slowly. The sudden drop was usually found to be due to a sudden blocking of the pulmonary artery, while in the gradual drop the emboli had blocked most of the pulmonary branches, and blood clots had formed around them. Respiration was unaffected until blood pressure began to decrease. Then it usually increased in both rate and amplitude. The blood pressure usually reached zero before respiration ceased.

At autopsy in every instance in which death had been produced by the emboli, the pulmonary circulation was found to be almost completely obstructed. Depending on the size of the emboli in some experiments, the pulmonary artery or the two branches were blocked; in others the occlusion occurred in the smaller branches. When the blood pressure had decreased slowly and venous pressure had increased considerably, many of the emboli sent into the circulation toward the end of the experiment were found in the right ventricle or vena cava.

The position assumed by the emboli in the pulmonary system in relation to the time of the injection was fairly uniform. As would be anticipated, the first emboli passed were usually found in the larger

branches of the pulmonary artery, and the first two or three emboli were found in the upper branch of the left branch of the pulmonary artery or in the pulmonary branch going to the largest lobe of the right lung. The positions of the rest of the emboli were never uniform.

As it was found impossible to produce sudden death by emboli in normal dogs without almost complete obstruction of the pulmonary circulation, the procedure was repeated using animals with a greatly depressed circulation. In a few experiments the animal was subjected to several hours' anesthesia before the emboli were used; other dogs were practically moribund with distemper. In all these animals the blood pressure was low (Protocol 2). The results in these experiments did not differ from those for which normal dogs were employed. Death was not produced until obstruction of the pulmonary circulation occurred.

It was deemed possible that general anesthesia was a factor. To obviate this, in a small series of animals the operative procedures were done under local anesthesia. The results were the same as when ether was employed (Protocol 3).

In a few experiments the emboli were sent in under sterile conditions. When very many emboli were employed the animal either died on the table or a short time afterward, or developed infarction of the lungs. When only a few emboli were employed the animal was not affected (Protocol 4).

Death from pulmonary embolism usually takes place in relatively strong patients at the time they attempt to leave the sickroom at the beginning of convalescence. They are usually active at the time of death. To simulate this condition a strong animal was fasted for several hours and the emboli passed into the circulation immediately after a period of intense exercise. The results of this experiment were also negative (Protocol 5).

EXPERIMENTAL.

Protocol 1.—Mongrel, male; weight 7 kilos.

December 22, 1916. 8.45 a.m. Animal etherized. The apparatus was arranged to record carotid blood pressure and respiration. Right femoral vein exposed. Normal record taken, beginning at 9.10 a.m. Emboli of paraffin, 4 cm. in length and 0.5 cm. in diameter, were inserted into the right femoral vein as follows:

Time.	Embolus No.	Color of embolus.
a.m.		
9.21	1	Lavender.
9.22	2	Blue.
9.22½	3	Red.
9.23	4	Pink.
9.24	5	Yellow.
9.24½	6	Orange.
9.25	7	Green.
9.25½	8	Lavender and yellow.
9.26½	9	Green and yellow.
9.27	10	Red and yellow.
9.28	11	Mixed, several colors.

The blood pressure was affected as each embolus passed through the heart. Later, blood pressure began to decrease, the dog dying at 9.47 a.m. Fig. 1 gives the kymograph record of blood pressure and respiration, and Figs. 2 and 3 give the size and location of emboli.

Protocol 2.—Bulldog, female; weight 18.6 kilos.

December 27, 1916. The animal was in very poor condition. 9.40 a.m. Etherized. The apparatus was arranged to record blood pressure and respiration. A small amount of blood was withdrawn. The right common iliac vein was exposed, as was also the right femoral vein. A blood vessel clip was placed on the iliac vein, and after the contributary veins were gently crushed with a hemostat, blood serum taken from the blood which had been withdrawn was injected into the injured veins. The same process was repeated with the left external jugular vein. Clots soon formed in each vessel. 10.37 a.m. Blood pressure 60 mm. 10.44 a.m. The clamp was removed from the jugular vein and the clots were swept into the circulation. The blood pressure decreased 12 mm. but soon returned to normal. 10.48 a.m. The clamp was removed from the iliac vein. The blood pressure immediately fell and gradually decreased until it reached zero at 10.54 a.m. Fig. 4 gives the kymograph record of the blood pressure and respiration.

Autopsy was performed immediately. There was a large clot, 3.5 by 1 cm.,

in the pulmonary artery and extending into the right ventricle. This practically completely obstructed the artery. The left branch of the pulmonary artery was empty, but most of the terminal branches of the right branch of the artery were blocked with small blood clots.

Protocol 3.—Collie, female; weight 9.3 kilos.

May 24, 1916. The right femoral vein was exposed under local anesthesia with sterile technique. Ten emboli of paraffin, varying in size from 2.5 to 3.5 cm. long and 0.5 cm. in diameter, were inserted into the vein. The time used in putting the ten emboli into the circulation was 10 minutes. The animal showed no symptoms referable to the emboli. The pulse and respiration remained normal. The animal remained in good condition until May 27, when it developed a marked dyspnea and respiratory grunt. It was bled to death under ether.

Autopsy was performed immediately. The recent femoral wound contained a small hematoma but was not infected. The lungs contained many hemorrhagic areas of infarction measuring 0.5 to 2 cm. in diameter. On the upper anterior surface of the lower right lobe was a small area of marginal emphysema. It was possible to palpate the emboli in the pulmonary artery. There were no emboli in the heart. It was impossible to identify all the emboli as some were broken into two or more pieces. The right branch of the pulmonary artery was completely blocked with the exception of the very small branches. The main branch of the left branch of the artery was completely blocked. The center of each lobe of both lungs was necrotic.

Protocol 4.—Young mongrel, male; weight 9 kilos.

December 14, 1915. The animal was etherized and the right femoral vein exposed, with sterile technique. Four emboli, about 3 cm. long and 0.5 cm. in diameter, were inserted into the vein. The animal recovered quickly from the operation and has been in excellent health up to the present time, 15 months after operation.

Protocol 5.—Old bulldog, male; weight 8.15 kilos; very pugnacious.

March 27, 1917. The animal had been fasted for 80 hours previous to the beginning of the experiment. About 100 cc. of blood were removed and set aside to clot. Under local anesthesia the right femoral vein was exposed and clamped with a blood vessel clamp. After the vein had been traumatized with a hemostat some of the animal's own blood serum containing small clots was injected. In a short time the vessel was filled with clots. The animal was then exercised for 5 minutes, after which the clamp was removed from the vessel and the clots were swept into the circulation. The respiration and pulse did not change. The process was repeated with the left external jugular vein. These results were also negative. 1 hour later the animal was etherized and bled to death.

Autopsy was performed immediately. Many of the terminal arteries in every lobe of the lungs were filled with clots. It was estimated that about half of the pulmonary circulation had been occluded.

Figs. 5 and 6 are kymograph records obtained in two similar experiments.

SUMMARY.

Emboli made of paraffin and the animal's own blood were sent into the venous circulation of dogs. Death did not occur until the pulmonary circulation was practically occluded. The results were the same whether the blood pressure of the animal was normal or depressed by ether or disease and whether the procedure was carried out under ether or local anesthesia.

EXPLANATION OF PLATES.

PLATE 35.

FIG. 1. Kymograph record of blood pressure and respiration in Protocol 1. Time in minutes and seconds. Normal blood pressure 90 mm. of mercury. Note the drops in the blood pressure as the emboli pass through the heart. The break in the record covers a space of 3 minutes. Note the increase in respiratory movements as the blood pressure falls.

FIG. 2. Photograph of emboli used in Protocol 1, which were recovered at autopsy. Actual size.

FIG. 3. Drawing showing the pulmonary artery and branches. The numbers designate the positions at which the emboli used in Protocol 1 lodged.

PLATE 36.

FIG. 4. Kymograph record of blood pressure and respiration in Protocol 2. Time in minutes and seconds. Normal blood pressure 60 mm. of mercury. At the signals the clots of the animal's own blood were allowed to enter the circulation. Death was due to a large clot from the right iliac vein which completely blocked the pulmonary artery (Signal 2).

FIG. 5. Kymograph record of blood pressure and respiration. Time in minutes and seconds. Normal blood pressure 100 mm. of mercury. Each signal marks the passage of an embolus of a blood clot of the animal's own blood from either the left jugular or the right iliac veins. The clots were made as described in the text. Note the drops in the blood pressure after the entrance of each embolus. The break in the record covers a space of 6 minutes. At autopsy both branches of the pulmonary artery were found to be blocked with the clots formed in the veins.

FIG. 6. Kymograph record of blood pressure and respiration. Time in minutes and seconds. Normal blood pressure 125 mm. of mercury. The vagi were sectioned. The signals mark the time of the insertion of the paraffin emboli (4 by 0.5 cm.) into the right femoral vein. Note that the emboli cause the small drops in the blood pressure even after section of the vagi. Death was not produced until the pulmonary artery and right ventricle were blocked.

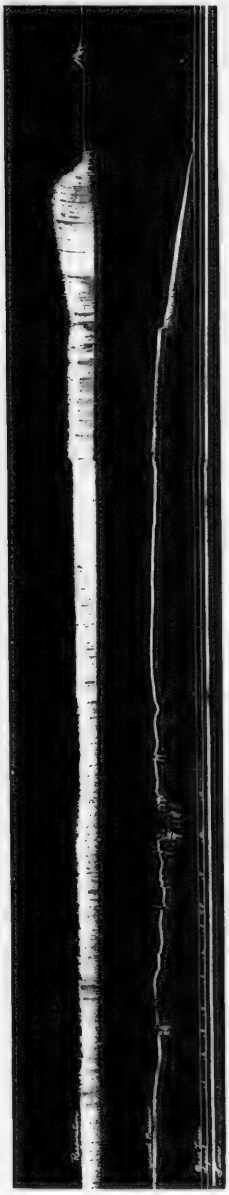


FIG. 1.

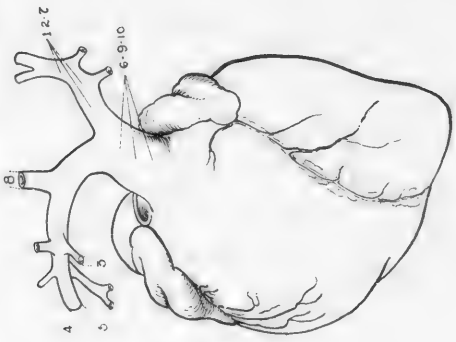


FIG. 3.

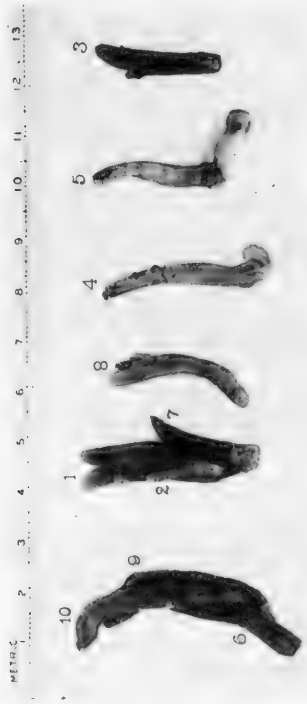


FIG. 2.

(Mann: Pulmonary embolism.)

FIG. 4.

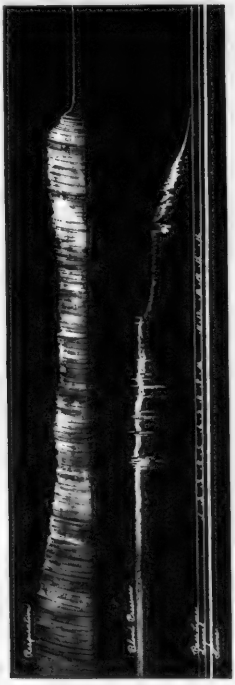


FIG. 6.

FIG 5.

(Mann: Pulmonary embolism.)

THE VISCOUS METAMORPHOSIS OF THE BLOOD PLATELETS.

By J. HOMER WRIGHT, M.D., and GEORGE R. MINOT, M.D.

(*From the Pathological Laboratory of the Massachusetts General Hospital, Boston.*)

PLATE 37.

(Received for publication, April 9, 1917.)

The phenomenon of agglutination and fusion of the blood platelets into glassy masses and strands, as observed in films of wet blood between a slide and cover-glass, was first described by Hayem (1) in 1878. Some years later, it was more exactly described by Bizzozero (2) and named "viscous metamorphosis" by Eberth and Schimmelbusch (3). It occurs during the process of coagulation and is well marked at the time of the first appearance of the fibrin threads. It does not take place if sodium oxalate or certain other salts have been added to the blood during the bleeding in sufficient amount to prevent coagulation and if other conditions are favorable. Mosen (4) in 1893 was the first to show that platelets from such a non-coagulating blood, freed from blood elements by centrifuging, do not fuse with one another, but retain their separate identity. They appear when in suspension in isotonic salt solution and observed with medium powers between a cover-glass and slide, as refringent, glistening granules or round bodies of a diameter varying approximately from one-fourth to one-half that of a red blood corpuscle (Fig. 1); they are not clumped but uniformly distributed and remain so until they disintegrate. We have found, however, that such platelets normally have not lost the ability of metamorphosis, but that they undergo the characteristic changes within a few minutes if serum or certain more or less closely related substances are added to the suspension. As observed under the conditions just mentioned, the platelets begin their metamorphosis by aggregating and agglutinating in masses and strands which increase in size up to a certain point within a few minutes. Then within a few more minutes, the plate-

lets in these masses and strands lose their high refrangibility, seem to fuse one with another, and their separate identity becomes lost or obscure. The masses and strands assume thereby the appearance of a more or less homogeneous, glassy, refringent substance in which refringent granules are usually present. During this transformation or viscous metamorphosis some contraction of the volume of the masses and strands may occur and by this contraction in volume, bands and strings of hyaline material may be produced, which unite masses of platelets in the process of transformation or already transformed. The peripheral or marginal portions of the masses and strands are usually more hyaline than the central parts and the marginal outlines are finely wavy and denticulate.

This metamorphosis of the isolated platelets in the presence of serum has been briefly described in recent papers by Lee and Robertson (5) and by Minot and Lee (6) in which also some results of experiments with other substances are noted. The present paper is based on further experiments on this line in the endeavor to determine the nature of the substance in the serum which causes or permits the metamorphosis to take place.

It should be noted that this metamorphosis is to be distinguished from simple agglutination of platelets without fusion, which may be caused by certain substances. The microscopic appearances of a weak simple agglutination are shown in Fig. 2. Here the agglutinated platelets retain their separate identity.

Figs. 3, 4, and 5 show the appearances of suspensions of platelets similar to that shown in Fig. 1; the suspensions have undergone the viscous metamorphosis.

Lee and Robertson (5) have shown, and it may be emphasized here, that this phenomenon of agglutination and fusion of platelets associated with coagulation is to be distinguished from agglutination and lysis of platelets caused by antiplatelet serum.

The phenomenon seems to begin when the first signs of clotting are seen and is thus perhaps the first demonstrable sign of the clotting process. This has been our experience with not only normal blood, but blood with delayed coagulation time, as from cases of hemophilia, pneumonia, and jaundice, no matter whether observed in whole blood in glass tubes, or from cloudy plasma (plasma relatively rich

in platelets) obtained from centrifugalized whole blood in iced paraffined tubes. In the abnormally slow coagulating cloudy plasma one may observe that clotting begins near the edge of the tube and microscopic preparations from this portion of the plasma will show the fusion of the platelets, while material obtained at the same time from the still fluid portion will show the platelets free.

Method.

Rabbit and human platelets were used. The blood was drawn from the rabbit's ventricle or from the veins of man by means of a needle and syringe. Before drawing the blood, a sufficient quantity of freshly prepared 1 per cent solution of sodium oxalate in 0.9 per cent sodium chloride solution was placed in the barrel of the syringe to constitute at least one part to nine parts of blood. Sometimes the needle and barrel of the syringe were coated with paraffin. In order to obtain platelets that are unaltered and suitable for studying the viscous metamorphosis, it is important that the needle enter the ventricle or the vein at the first thrust so as to avoid contamination with the tissue juice as much as possible, and that the blood should flow quickly and freely into the syringe and be rapidly mixed with the oxalate solution. If these conditions are not observed, the platelets are likely to undergo more or less fusion before isolation or to be otherwise altered so that they will not undergo the characteristic metamorphosis. Slight alteration in them, inappreciable in any change in microscopic appearance and often impossible to control, seems to render them incapable of undergoing the changes. Many times we have isolated platelets in apparently intact and unaltered condition which showed themselves but feebly active or inert.

On the other hand, platelets may be quite grossly altered without essentially affecting their activity as cytozyme (Bordet and Delange (7)) or as thromboplastin (Howell (8)). Even after they have undergone their metamorphosis we have found them capable of acting efficiently as factors in coagulation of the blood.

The platelets were separated from the other elements of the blood by centrifuging at low and high speeds, and were at least twice sus-

pended and thrown down in 0.9 per cent sodium chloride solution. Unaltered platelets, thus isolated and washed, form a white, semi-fluid, somewhat sticky sediment at the bottom of the centrifuge tube and they are readily miscible with salt solution by agitation with the pipette. If the sediment of the platelets forms an elastic coherent mass or masses, some metamorphosis has already occurred and such platelets are not suitable for use.

The isolated platelets in salt solution retain their ability to undergo the metamorphosis for a period varying from usually a few hours to rarely 2 or 3 days. This ability seemed to be retained longer at room temperature than in the ice box or incubator, and also if a very small amount of oxalate had been added to the salt solution.

The blood elements used in studying this phenomenon of agglutination and fusion of the blood platelets were usually obtained from rabbits. Most of the experiments were also done with human material. No differences in the results were noted when the platelets came from man or rabbit and the various other substances came from the same animal or a different animal of the same or different species. It was found rather more difficult to obtain suitable platelets from the arm veins of normal men than from the hearts of normal rabbits.

In testing the effect of the various fluids and solutions on the isolated platelets a thick suspension in 0.9 per cent sodium chloride solution was found best to use. This was prepared usually by mixing the sediment of platelets at the bottom of the centrifuge tube with three or four drops of the solution. It was found important that the platelets should be well mixed with this salt solution so as to be separated from one another and that they should be used in a very thick suspension as described. A very thin suspension of platelets was found undesirable for it seemed possible for the platelets to go through their metamorphosis in but tiny clumps or singly, so that the phenomenon was not clearly observable. Again, very compact masses of platelets were not used both on account of a possibility of an excess of platelets altering the results and also because the substance affecting the platelets may not penetrate to the center of the mass so that the metamorphosis will be observed only about the edge of the mass.

After a satisfactory suspension of blood platelets was obtained the action of various substances upon them was observed by mixing from one to three small drops of the materials in question with a very small drop (obtained from capillary glass tubing) of the very thick platelet suspension on a glass slide, covering with a cover-glass, and observing microscopically.

Effect of Serum, Thrombin, Calcium, and Antithrombin and Thromboplastin.

Serum was capable of producing well marked metamorphosis of the platelets usually in 2 to 7 minutes. Sometimes the change did not occur until after longer periods, up to 20 minutes or more. The time required varied both with different lots of platelets and with different sera. It was not clearly dependent on the age of the serum, although it may be said that sera a few hours old often seemed more effective than when very fresh, or sera 10 or more hours old.[1]

This power of serum was found present on some occasions in dilutions (made with 0.9 per cent salt solution) of 1: 10, on other occasions in dilutions as high as 1: 50. The greater the dilution the longer it took for the metamorphosis to occur. When calcium was added to the serum dilutions so that they contained 0.05 per cent calcium chloride it accelerated the metamorphosis and caused it to appear with those dilutions in which only slight agglutination had occurred. It is to be noted that with the higher dilutions that caused metamorphosis relatively fewer platelets seemed to be transformed than with the lower dilutions, suggesting a quantitative action of the substance that metamorphoses platelets.

Table I is a typical protocol showing the effect of dilution of serum with and without calcium.

With serum this phenomenon can be demonstrated macroscopically. If several small drops of a thick platelet suspension are added to 1 cc. of serum they will be at first evenly distributed through the serum giving it a cloudy appearance. When the metamorphosis has occurred they will be found clumped in the bottom of the tube and

[1] Rarely a serum produced no effect.

TABLE I.

Serum dilution with 0.9 per cent sodium chloride solution.	One drop of 0.9 per cent sodium chloride solution.		One drop of 0.5 per cent calcium chloride solution in 0.9 per cent sodium chloride solution.	
		min.		*min.*
0	F*	2	F	2
1 : 6	F	5	F	2
1 : 15	F	8	F	4
1 : 25	F	13	F	6
1 : 40	F	20	F	12
1 : 60	A	15	F	18
1 : 80	A?	20+	F	20
1 : 100	0		F	25
1 : 120	0		A	20
1 : 150	0		A?	25
1 : 200	0		0	

* The figures in the table give the time in minutes for definite fusion (F) (viscous metamorphosis) or slight agglutination (A) of the platelets to take place under the given conditions.

upon shaking the tube the platelets will be seen to appear as small granular masses in a clear serum.

When platelets were treated with distilled water some portion of them went into solution and they lost their glistening appearance. Such a solution has definite thromboplastic (cytozymic) action in contrast to salt solution in which platelets have stood and been removed. The remaining shells of the platelets found after the platelets had been exposed to water were slightly clumped by serum, but a true metamorphosis did not take place.

Serum heated to 56°C. for half an hour or serum heated up to 60°C., at which temperatures thrombin is destroyed, caused no metamorphosis of platelets but did permit an agglutination of the platelets, and on some occasions seemed to allow a very slight lysis of the platelets. Platelets so treated, however, could still be metamorphosed by unheated serum, though possibly not so actively as when added directly to serum. This heated serum contained, as far as elements of coagulation are concerned, essentially only antithrombin and calcium.

From the above experiments we were inclined to believe that the viscous metamorphosis of platelets was due essentially to thrombin

aided by calcium. This, however, does not seem to be true, not only because of the results with "serozyme" (7), egg white, etc., described later, but also because repeatedly solutions of very active pure crystallized thrombin, prepared by Howell's method (9), did not produce metamorphosis of the platelets; although a slight slow clumping sometimes occurred there was never any fusion. If, however, calcium was added to the pure thrombin solution the platelets were often quite rapidly agglutinated into little ball-like masses but there was no metamorphosis.

Centrifuged, clear, oxalated plasma heated to 60°C. contains antithrombin but essentially no other coagulating element. Such antithrombin produced usually a very slight agglutination but no metamorphosis of the platelets. If, however, calcium was added, an agglutination, and possibly on some occasions, slight lysis occurred exactly as occurred but to a greater degree with the heated serum which contained the same two elements, antithrombin and calcium. When thrombin and antithrombin were mixed and allowed to stand 1 minute or 1 hour, the effect of the mixture on the platelets was the same as with thrombin or antithrombin alone. Antithrombin incubated with serum for a longer or shorter period did not have any definite effect on the ability of the serum to metamorphose platelets.

When pure thrombin and antithrombin from serum heated to 60°C. were mixed for 1 minute or 1 hour and calcium was added before or after the substances had remained in contact, the action of the mixture on the platelets was the same. Such a mixture produced a marked clumping of the platelets, faster and more marked than with thrombin and calcium or antithrombin and calcium. Occasionally this mixture also slowly caused a true metamorphosis of the platelets. Usually this was found not to be the case, but rather that besides the agglutination, a pseudo- or abortive metamorphosis occurred. This pseudo- or abortive metamorphosis consisted simply of a very marked agglutination with apparently a slight lysis of the platelets. On staining with Wright's blood stain the individual platelets in these masses were distinct and fairly sharply outlined and did not tend to form a homogeneous fused mass as was seen when true viscous metamorphosis occurred. Platelets so altered or platelets simply agglutinated by antithrombin and calcium or thrombin and

calcium were still capable of being metamorphosed with serum, but apparently not quite so effectively as unaltered platelets. This indicates that the process of agglutination of the platelets is to be distinguished from the viscous metamorphosis, though agglutination of some type is an integral part of the viscous phenomenon.

When mixtures of pure thrombin and calcium with antithrombin from oxalated plasma rather than from serum were mixed with platelets we never observed a true metamorphosis. Such a mixture did allow definite agglutination and at times a weak pseudo- or abortive metamorphosis.

Thromboplastin in various forms (fresh tissue juice, cephalin, or coagulen[2]) did not apparently influence the platelets either alone or when added in reasonable amount to any of the various substances or mixtures in the experiments outlined above or below.

Effect of Serozyme, Oxalated Plasma Plus Thrombin, and Fibrinogen.

Oxalated plasma whether dialyzed or not had, of course, no effect on the platelets, and when oxalate was added to any of the substances referred to above or below, it either prevented or weakened (if relatively little was added) their action on platelets. When the amount of calcium that clotted most quickly a given oxalated plasma was mixed with it and platelets were added at once to the mixture, which was then observed under the microscope, the platelets were seen to undergo their typical transformation. They began to do so as the first signs of coagulation appeared. In such a mixture, however, they completed their metamorphosis more slowly than under more favorable conditions. This we attributed to the presence of oxalate, for when a plasma had been dialyzed to remove the excess of oxalate, and calcium was then added to it with platelets, the latter elements underwent their metamorphosis more rapidly than when the control undialyzed oxalated plasma was used. This was true whether fresh plasma or dried plasma a year old, but freshly dissolved, was used.

The fluid resulting from the defibrination of a clot, formed with oxalated plasma and an optimal amount of calcium, is called by

[2] Commercial preparation made by the Society of Chemical Industry, Basle, Switzerland.

Bordet and Delange (7) "serozyme." When this is freshly prepared it often contains free thrombin. On standing some of the thrombin seems to combine with antithrombin and other portions of the thrombin become perhaps altered in some manner so that free thrombin cannot be demonstrated. A good serozyme, *i.e.*, one without free thrombin, as evidenced by its inability to clot fibrinogen in 24 hours, is to be obtained from a very clear rather than a cloudy plasma. Serozyme and cytozyme (tissue juice, thromboplastic substance) and calcium form thrombin in Bordet and Delange's terminology as is shown by the fact that after this mixture has stood for about 6 minutes it clots fibrinogen in 1 to 2 minutes, while suitable controls form no thrombin. Serozyme was found to be usually as active in causing the metamorphosis of platelets as serum, and its activity increased if a mere trace of calcium was added to it. It seemed to make little difference whether the serozyme contained free thrombin or not. Though rather definite in some instances but not in all, a serozyme containing free thrombin, prepared from cloudy plasma, seemed to be not quite so active in causing the platelet metamorphosis as a serozyme that had no free thrombin. This would suggest that the substance causing platelet metamorphosis had been partly used up in transforming the platelets in the cloudy plasma.

The following observations on the action of a mixture of Howell's pure thrombin and oxalated plasma were made. When these two substances, which form a clot in about 3 minutes, were mixed, and platelets added at once, the typical viscous metamorphosis of the platelets occurred usually in 2 to 3 minutes, provided apparently that the oxalated plasma was not oxalated to contain above about 0.1 per cent of oxalate. On some occasions, usually if the oxalation was excessive, only a rather marked agglutination or pseudometamorphosis of the platelets occurred about the fibrin strands, which suggests, as do the experiments with the mixture of thrombin, serum antithrombin, and calcium, that this phenomenon is an abortive type of true metamorphosis.

When thrombin and dialyzed oxalated plasma were used the platelets were always metamorphosed.

We may note that presumably all the substances mentioned before which produced this change in the platelets contained at least

traces of ionized calcium. Perhaps, with the rearrangement of the colloidal substances which occurs when thrombin clots the fibrinogen of the oxalated plasma, some calcium is temporarily set free. That some substance, as well as possibly calcium, is set free in this reaction which metamorphoses the platelets, is suggested by the above experiments, and that this substance then rapidly becomes relatively inactive is suggested by the following observations.

The fluid resulting from the complete clotting of oxalated plasma with thrombin seldom permitted the viscous metamorphosis of the platelets; sometimes a very slow and poor but true transformation occurred. This result was probably due to the oxalate, because the fluid resulting from thrombin and dialyzed plasma did permit, though always slowly (20 minutes) the transformation of the platelets. Such delayed reactions are in contrast to the relatively rapid effect on platelets during the clotting of plasma with thrombin. If, however, calcium was added to either of the fluids resulting from clotting dialyzed or undialyzed oxalated plasma with thrombin, they then not only always permitted a metamorphosis of the platelets, but also a rapid one (5 minutes). This may have been partially due to the effect of calcium on the oxalate, besides its evident accelerating action, as well as perhaps to some change that occurred in these fluids when the calcium acted on the prothrombin present in them. That these fluids contained prothrombin is evident, because this substance was in the oxalated plasma and there had been no calcium added to transform it to thrombin.

Magnesium sulfate plasma and thrombin acted essentially like oxalated plasma and thrombin.

Solutions of fibrinogen, prepared according to a modification of Hammarsten's method, were found to have no effect on the platelets. These fibrinogen solutions unfortunately were not absolutely free of prothrombin, but contained enough to clot in 3 hours with the optimal amount of calcium. The results of experiments with thrombin and this fibrinogen were essentially like those with thrombin and dialyzed plasma, except that the metamorphosis of the platelets was never marked even when calcium was added to the serum from the coagulation of this fibrinogen by thrombin. It would seem that, during the process of clotting fibrinogen there occurred, with the

rearrangement of the colloidal particles, a setting free of some substance allied to serozyme which was very active on the platelets, and, with the completion of such coagulation, relatively little substance was left free, though addition of calcium, perhaps by its property of aggregating colloidal particles, would permit the substance causing platelet metamorphosis to become more active.

Absorption from Serum of the Substance Causing Metamorphosis.

Experiments were conducted to determine whether one could absorb from serum the substance that transforms platelets. Barium sulfate absorbs serozyme-like substances as shown by Bordet and Delange and Lee and Vincent (10), but it does not seem to affect formed thrombin in Bordet and Delange's sense. We found that when 1 cc. of serum was treated with 0.4 cc. of washed barium sulfate for 2 hours and then centrifuged clear, that the serum had lost its power of metamorphosing platelets even after calcium was added.

The effects of adding large amounts of cephalin to serum were next studied. A relatively large amount of cephalin in solid form was added to serum so that some of it dissolved in the serum. When this mixture was then centrifuged, so that the resulting fluid was clear, it was found to have lost its ability to metamorphose platelets, though sera so treated were able to clot fibrinogen. When calcium was added to such serum it did not restore the ability to metamorphose platelets. This effect of cephalin was obtained in but a third of the numerous times it was tried on 6 different days. It seemed that if the sera could be centrifuged so that they were clear after treatment with cephalin, this substance was much more apt to be effective than if the centrifuged sera remained at all cloudy. Sera, when somewhat diluted with salt solution to which thick suspensions of platelets were added and then centrifuged clear after the platelets were fully agglutinated and fused, sometimes lost their ability to further metamorphose platelets, as was the case with sera treated with cephalin. Undiluted sera could not be affected in this way, probably because too few platelets were added. Similar results were obtained upon treating sera with cholesterolized syphilitic antigen as with cephalin and platelets. We have found no explanation for these inconstant results.

It was thought that the power of syphilitic serum to metamorphose platelets might be more easily exhausted with syphilitic antigen than normal serum, in view of the inhibitory effect of syphilitic serum in Bordet and Delange's method of forming thrombin as shown by Hirschfeld and Klinger (11). No such difference, however, could be detected.

Starches of various kinds, agar, gelatin, gold chloride, cholesterol, and chloroform in various strengths, and various other substances did not, after being mixed with serum for 2 hours, alter its ability to produce this metamorphosis. Neither did these substances alone metamorphose the platelets.

Ability of Serum Globulin and Egg White to Metamorphose Platelets.

In order to determine whether the power of serum to metamorphose platelets resided in the globulin or albumin fraction, the following experiment was made. Serum was dialyzed against distilled water. When a small amount of precipitate had appeared at the bottom of the dialysis sac, the serum was still active. Later, when a considerable amount of precipitate occurred in the dialysis sac, the fluid with or without calcium was inactive on the platelets. The dialysate was also inactive. The precipitated globulin, when redissolved in salt solution with the aid of a trace of dilute alkali, which of itself did not affect the platelets, caused a feeble metamorphosis of the platelets. However, when calcium was added to this solution a definite active metamorphosis of the platelets was produced with it.

Egg white alone or shaken with isotonic salt solution had a marked effect on the platelets. It caused them to be rapidly metamorphosed and often seemed to act more efficiently than serum. This result suggested that egg white contains a serozyme-like element. Experiments showed this to be the case, for it was found that it could take the place of serozyme in the formation of thrombin by the method of Bordet and Delange. Hirschfeld and Klinger (12) have recently also detected a serozyme-like action of egg white. Egg white alone apparently does not clot fibrinogen and therefore does not contain formed thrombin.

Numerous pure proteins,[3] lactalbumin, gliadin, legumin, vignin, edestin, and cottonseed globulin, and also milk with or without calcium had no effect on the platelets.

Metamorphosis of Platelets from Abnormal Bloods.

The platelets from cases of hemophilia, pneumonia, purpura hæmorrhagica, purpura rheumatica, and obstructive jaundice were transformed by either their serum or normal serum in the same way and essentially in the same time as normal platelets. In this connection it is to be noted that hemophilic platelets are, however, defective in their cytozymic action and that they do not begin to fuse until the blood shows visible signs of clotting, as has been shown by Minot and Lee (6). On this account the metamorphosis of hemophilic platelets and normal platelets was studied with those substances causing metamorphosis of normal platelets, derived from normal and hemophilic blood, but no real differences in the character or rate of metamorphosis could be detected.

SUMMARY AND CONCLUSIONS.

From the experiments above described it is evident that the viscous metamorphosis of the blood platelets in shed blood is intimately associated with the early stages of coagulation and that the presence of calcium is a very important element, though perhaps not absolutely necessary. No constant differences in this phenomenon could be detected with platelets or coagulating elements from the blood of normal or diseased individuals or from man or rabbits.

All the substances, with the exception of egg white, causing this phenomenon were derived from the blood either during the alteration of fibrinogen or after it had been acted on by thrombin. It seems that the substances or mixtures capable of producing the metamorphosis are especially those associated with the early stages of coagulation or capable in the test-tube of forming or liberating that active coagulating element known as thrombin.

The substance in serum that is capable of metamorphosing platelets seems to be attached to the globulin fraction rather than the

[3] These proteins were kindly furnished by Dr. W. Denis.

albumin fraction and is destroyed by heat at temperatures which destroy prothrombin, thrombin, and serozyme, and precipitate fibrinogen.

The reaction is not caused by pure thrombin or a mixture of pure thrombin and calcium, though substances causing the metamorphosis are intimately related to thrombin. The metamorphosis seems to be caused by serozyme-like substances as shown both by the fact that barium sulfate absorbs the power of serum to cause the reaction and that a serozyme-like substance is probably to be recognized in all the substances or mixtures, including egg white, causing this change in the platelets, except perhaps when thrombin and fibrinogen are reacting. It is to be noted that in serozyme are contained antithrombin, calcium, and potential thrombin, and that a combination of these isolated factors mixed together occasionally allowed the viscous metamorphosis to occur and not infrequently an abortive metamorphosis.

The pseudo- or abortive metamorphosis caused by the mixture of pure thrombin, antithrombin, and calcium may be interpreted on the supposition of a close approximation but not a real reproduction of the colloidal state known as serozyme. That such a reaction is related to real viscous metamorphosis is suggested, because it sometimes occurred with the above mixture and when thrombin and heavily oxalated plasmas were reacting, rather than a real metamorphosis.

Simple agglutination of the platelets may occur independently of a viscous metamorphosis, though an agglutination of platelets is to be considered an integral part of the viscous metamorphosis phenomenon.

The inconstant results seen with the cephalin-treated sera are probably due to the fact that exactly the same mixtures were not obtained. The results with cephalin-soaked and platelet-soaked sera and with dilutions of sera suggest that the reaction of viscous metamorphosis of the platelets is quantitative.

BIBLIOGRAPHY.

1. Hayem, G., *Arch. physiol. norm. et path.*, 1878, v, series 2, 692.
2. Bizzozero, J., *Virchows Arch. path. Anat.*, 1882, xc, 261.
3. Eberth; J. C., and Schimmelbusch, C., *Virchows Arch. path. Anat.*, 1886, ciii, 39.

4. Mosen, R., *Arch. Physiol.*, 1893, 352.
5. Lee, R. I., and Robertson, O. H., *J. Med. Research*, 1915–16, xxxiii, 323.
6. Minot, G. R., and Lee, R. I., *Arch. Int. Med.*, 1916, xviii, 474.
7. Bordet, J., and Delange, L., *Ann. Inst. Pasteur*, 1912, xxvi, 657, 737.
8. Howell, W. H., *Am. J. Physiol.*, 1911–12, xxix, 187.
9. Howell, *Am. J. Physiol.*, 1910, xxvi, 453; 1913, xxxii, 264.
10. Lee, R. I., and Vincent, B., *Arch. Int. Med.*, 1914, xiii, 398.
11. Hirschfeld, L., and Klinger, R., *Deutsch. med. Woch.*, 1914, xl, 1607.
12. Hirschfeld and Klinger, *Biochem. Z.*, 1916, lxxvi, 145.

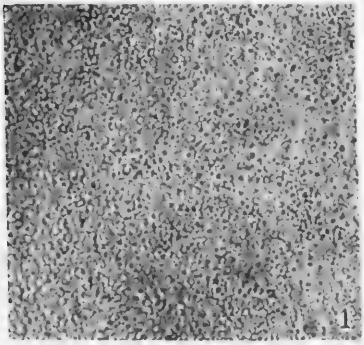
EXPLANATION OF PLATE 37.[4]

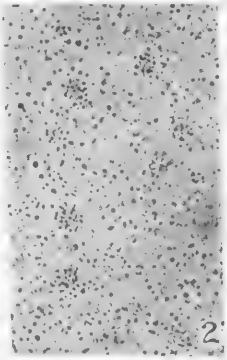
FIG. 1. Suspension of blood platelets in isotonic salt solution. × 300.

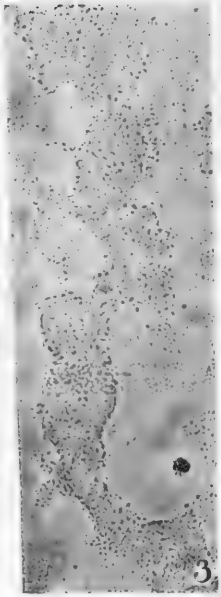
FIG. 2. Simple, weak agglutination of platelets. There are fewer platelets in this preparation than in those shown in the other figures. × 300.

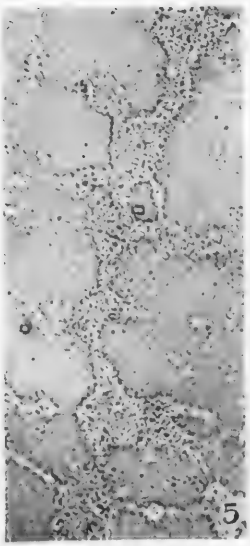
FIGS. 3, 4, and 5. Viscous metamorphosis of platelets. Essentially the same number of platelets was in these preparations as in the preparation shown in Fig. 1. × 300.

[4] The photographs were made by Mr. L. S. Brown.









(Wright and Minot: Viscous metamorphosis of blood platelets.)

STUDIES ON BACTERIAL ANAPHYLAXIS AND INFECTION.

By HANS ZINSSER, M.D., and JULIA T. PARKER.

(*From the Department of Bacteriology of the College of Physicians and Surgeons, Columbia University, New York.*)

Plates 38 and 39.

(Received for publication, April 19, 1917.)

Since anaphylaxis has now been clearly recognized as a phenomenon which depends upon an antigen-antibody reaction, it follows naturally that the fundamental principles underlying these manifestations should apply, subject perhaps to individual modifying factors, to all substances which on injection into the animal body induce specific antibody formation. By far the greater volume of work on anaphylaxis has been carried out with antigens consisting of animal sera, egg white, and other freely suspended proteins not enclosed in cells. And in the case of such serum anaphylaxis it has been possible to go far in the analysis of the mechanism of the phenomenon and its application to such practical problems as serum sickness and other clinical conditions referable to abnormal protein metabolism.

There has been relatively less progress, however, in the study of the hypersusceptibility incident to the treatment of animals with cellular antigens; that is, with bacteria, erythrocytes, and other cells. And yet these phenomena in the case of bacteria may reasonably be assumed to play an important part in the symptomatology and pathology of infectious diseases where there is a continuous liberation of fluctuating amounts of specific antigen in a body at first perhaps unsensitized, but later increasingly hypersusceptible, and perhaps repeatedly sensitized and desensitized during the course of the disease. Of these conditions we know very little, and correspondingly much speculation and reasoning from the analogies of serum anaphylaxis have been the bases of many inferences and theoretical suggestions.

It is plain that when the antigen employed is a cellular one we are necessarily dealing with a phenomenon consisting of two phases. As integral parts of compact structures, the cell constituents cannot react as antigen with the cells and fluids of the invaded body until they have been liberated by cell disintegration. After that the process may be, and probably is, entirely similar to serum anaphylaxis. But before this state of affairs can be brought about it must be assumed that some preliminary reaction occurs in which the bacterial protein is set free from the cell. As to the nature of this reaction we have no certain knowledge. We cannot be sure at present, at least in our opinion, whether such a reaction is or is not accompanied by the direct formation of toxic cleavage products within the circulation such as those produced *in vitro* by Vaughan,[1] Friedberger,[2] and others, though this seems likely. Moreover, we do not know to what degree and under what circumstances such a process may be interfered with by intercurrent phagocytosis, and perhaps by bacterial agglutination and accumulation in the viscera, as demonstrated by Bull.[3]

The practical bearing of bacterial anaphylaxis on the course and symptomatology of infectious disease is of necessity very great. It has been suggested that the incubation time of infections may be in part dependent upon the gradual development of the specific sensitization of the tissues as antibodies begin to appear. The evidence adduced by the study of the isolated uterus, intestinal musculature, the perfused heart and lungs, and such experiments as those of Longcope[4] on the kidneys of rabbits points to the possibility that many of the insults offered various tissues, formerly attributed to specific preformed bacterial poisons, may be anaphylactic in nature. It is not impossible that a process of alternate sensitization and desensitization, either interrupted or continuous and gradual, may represent the dominating mechanism of injury in bacterial disease.

[1] Vaughan, V. C., Vaughan, V. C., Jr., and Vaughan, J. W., Protein split products in relation to immunity and disease, Philadelphia and New York, 1913.

[2] Friedberger, E., Series of papers in *Z. Immunitätsforsch., Orig.*, 1910 to 1913.

[3] Bull, C. G., *J. Exp. Med.*, 1915, xxii, 475.

[4] Longcope, W. T., *J. Exp. Med.*, 1913, xviii, 678.

We do not yet understand clearly the relation of the circulating antibodies to such processes. Do they aid in the intravascular mechanism for the liberation of toxic substances as suggested by Pfeiffer, Radziewsky, Wolff-Eisner, and more recently by Vaughan, Friedberger, and others? Or do they both hasten the removal of antigen by bactericidal and opsonic action and perhaps even protect the sensitized tissues by combining with the circulating antigen and diverting it from the sessile antibodies of the sensitized tissues? It would be interesting and important to know, moreover, whether or not there is a true bacterial hypersusceptibility in the sense that an animal treated with dead bacteria or bacterial products (as in vaccination) might pass through a preliminary period during which susceptibility to invasion by the living organisms or injury by their body constituents is increased.

All these problems await an analysis of bacterial anaphylaxis as thorough as that which has been made with antigens of non-cellular nature.

In order to approach the problem properly it is desirable to review briefly what has been done and how much of it may be accepted as needing no further inquiry.

The first investigators who systematically studied serum anaphylaxis naturally turned their attention, also, to anaphylaxis produced with bacterial protein. Thus Rosenau and Anderson[5] obtained definite results with extracts of various bacteria, using chiefly colon bacilli, extracts of yeast, of *B. subtilis*, and of typhoid bacilli. They injected relatively large quantities of the extracts subcutaneously into guinea pigs and, after periods of from 19 to 35 days, reinjected intraperitoneally or subcutaneously. They obtained reactions which they characterized as "mild," "marked," or "severe," but rarely observed acute anaphylactic death. Subsequently a large number of investigators turned their attention to the same problem and more or less divergent results were reported. The work was done chiefly by Kraus and Doerr,[6] Kraus and Amiradžibi,[7] Holobut,[8] Yamanouchi,[9] Delanoë,[10] Ascoli,[11] and Friedberger and his associates. It is hardly necessary

[5] Rosenau, M. J., and Anderson, J. F., *Bull. Hyg. Lab., U. S. P. H., 36*, 1907.
[6] Kraus, R., and Doerr, R., *Wien. klin. Woch.*, 1908, xxi, 1008
[7] Kraus, R., and Amiradžibi, F. S., *Z. Immunitätsforsch., Orig.*, 1909–10, iv, 607.
[8] Holobut, T., *Z. Immunitätsforsch., Orig.*, 1909, iii, 639.
[9] Yamanouchi, T., *Compt. rend. Soc. biol.*, 1909, lxvi, 531.
[10] Delanoë, M. P., *Compt. rend. Soc. biol.*, 1909, lxvi, 207, 252, 348, 389.
[11] Ascoli, M., *Compt. rend. Soc. biol.*, 1908, lxv, 611.

to go into a detailed historical review of the literature since the difficulties encountered, and the general conclusions drawn, may be summarized very simply. Most of the work done was carried out with definite prejudice in favor of complete analogy between serum hypersensitiveness and bacterial anaphylaxis. Kraus and Doerr, as well as Holobut and others, claimed that sensitization could be accomplished with regularity only if small doses of bacteria, *i.e.*, one one-hundredth of a loopful, were given daily for 10 days and reinjection was practiced about 3 weeks after the last injection with a relatively large amount washed up with and partly extracted in slightly alkalinized salt solution. It was found by a number of observers that the results were not uniform with different species of bacteria. It apparently has been very difficult, according to Müller, to sensitize with streptococci perhaps, as Müller suggests, because of the relative insolubility of these bacteria. Moreover, acute symptoms on reinjection in bacterial anaphylaxis have not been obtained with regularity and have been comparable to the acute shock in serum anaphylaxis in the smaller number of cases only.

Delanoë has denied the strict specificity of bacterial anaphylaxis, claiming that animals sensitized with typhoid bacilli reacted distinctly when reinjected with colon and paratyphoid A and B bacilli. This, however, may be true and yet fail to contradict the essential specificity of bacterial anaphylaxis as asserted by most investigators, since a group reaction might easily have accounted for his results, together with the toxic reaction so often noticed on first injection of any bacterial protein into guinea pigs.

Passive sensitization against bacterial protein was first reported by Kraus and Doerr and was further worked upon by Kraus and Amiradžibi. They injected guinea pigs with the serum of immune guinea pigs and rabbits, and 24 hours later injected the antigen intravenously or intraperitoneally, obtaining acute shock which sometimes killed the animals. Delanoë reported similar experiments and Yamanouchi and Ascoli suggested the utilization of passive anaphylaxis for the diagnosis of tuberculosis and typhoid fever.

Kraus and Amiradžibi also published experiments in which they obtained shock in guinea pigs into which they had injected typhoid bacilli mixed with the immune rabbit serum just before injection. These experiments are cited throughout the literature as pointing to the intravascular mechanism of bacterial anaphylaxis. Whatever may be our opinion concerning this question, and we will further discuss this in another place, the experiments of Kraus and Amiradžibi are inconclusive since they entirely disregard the not infrequent toxicity of normal and immune rabbit serum for guinea pigs, a disturbing factor which we encountered very early in our own work. In their protocols they record positive results only when the dose of serum was equal to or exceeded 0.5 cc. of rabbit serum and they confirmed the suspicion that serum toxicity was the true reason for the observed shock by stating that the active rabbit serum had lost its ability to give reactions when injected with bacteria after having been pre-

served for a month, a fact which our own investigations and the work of others have shown to be the case with sera toxic for animals of another species. The experiments of Kraus and Amiradžibi therefore, may be disregarded as having any bearing on the intravascular nature of bacterial anaphylaxis.

Nevertheless, from the work of other observers (Friedberger and Mita, Delanoë, and others) it seems beyond question that, just as one would expect, passive sensitization of guinea pigs with the sera of animals sensitized or immune to bacteria is possible. There is some discrepancy, however, between the results obtained by us and those reported by the observers mentioned in regard to the time necessary between injection of the antiserum and the development of the hypersensitiveness. This is a matter concerning which we will have more to say below.

As far, then, as reaction with the dissolved bacterial antigen is concerned there seems to be a complete analogy between serum and bacterial anaphylaxis except in one important and perhaps fundamental point, and this is the fact of primary toxicity of the bacterial protein. For, while the first inoculation of such antigens as horse serum, sheep serum, egg white, etc., produces no symptoms of injury in the injected animal, typhoid protein and that of many other bacteria if given in sufficient amounts lead even at first injection to severe illness and rapid or delayed death according to the variety of bacteria and the amount injected.

Indeed it is an observation recorded by Friedberger and Mita, by Seitz,[12] by Müller,[13] and by Doerr,[14] as well as occasionally made in our own experiments, that typical anaphylactic symptoms may follow immediately upon first injection of bacteria and bacterial extracts, if the amount given is sufficient. Müller particularly, has subjected this occurrence to systematic study. He finds that the chief difference between the reaction of the sensitized and the unsensitized guinea pig to intravenous injection of bacterial protein consists in the facts that the sensitized animal will show acute reaction to doses smaller than those necessary to produce the same result in the normal controls and that these smaller doses, which produce acute anaphylactic death in the sensitized, will produce a

[12] Seitz, A., *Z. Immunitätsforsch., Orig.*, 1912, xiv, 91.

[13] Müller, P. T., *Z. Immunitätsforsch., Orig.*, 1912, xiv, 426.

[14] Doerr, R., in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1913, ii, 1098.

more protracted illness and often death after the lapse of hours or days in the unsensitized. Müller interprets his work entirely in the sense of Friedberger's anaphylatoxin theory, in that he believes acute death to be due to the formation in the blood stream of a poison resulting from the reaction between plasma and bacteria, a process which is therefore more rapid in the sensitized animals in whose blood stream antibodies are present in relatively higher concentration.

These considerations bring the problem of bacterial anaphylaxis into intimate relationship with questions concerning the nature of the toxic properties of such microorganisms as the typhoid, paratyphoid, and plague bacilli, the cholera spirillum, and other bacteria, which unlike diphtheria and tetanus, do not secrete true toxins or exotoxins. Friedberger's theory would both explain anaphylactic poisoning with bacterial antigens and relegate to the chapter of discarded theories the older endotoxin conception of Pfeiffer which assumed the existence of specific preformed intracellular poisons liberated mechanically by disintegration of the cell body.

As a matter of fact, the entire problem of endotoxins is one which calls for reexamination in that the knowledge gained of recent years has opened a number of alternative explanations for the primary toxicity of such bacteria as the typhoid bacillus. Briefly summarized they are: (1) The actual intracellular existence of specific endotoxins in the sense of Pfeiffer[15] (toxalbumin). (2) The production of toxic split products in the animal body from the bacterial protein by proteolytic cleavage brought about by non-specific serum protease (Jobling and Petersen[16]) or by the cooperation of antibody and alexin (Friedberger). (3) The absorption of antienzymes by the bacteria with consequent activation of the serum protease which then splits off toxic substances from the plasma protein (Jobling and Petersen[17]). (4) The presence of non-specific toxic substances in the bacterial cell body, of the nature of peptones, primary and secondary albumoses, etc., which are liberated by lysis from the bacterial cell after cell death. This conception would differ from that of Pfeiffer in that the intracellular substances are conceived as in no sense specific toxic proteins, but rather entirely non-specific constituents repre-

[15] Pfeiffer, R., *Z. Hyg.*, 1892, xi, 393.
[16] Jobling, J. W., and Petersen, W., *J. Exp. Med.*, 1914, xx, 452.
[17] Jobling and Petersen, *J. Exp. Med.*, 1914, xx, 321.

senting the type of poisons conceived as proteolytically produced from the antigen by Vaughan and others. This last view, though hitherto not particularly considered, should nevertheless in our opinion be regarded as at least a possible explanation for a part of the toxic manifestations resulting from the injection of bacteria of this class. Moreover, such a possibility is suggested by the fact that bacterial protein is relatively poor in antigenic properties. Doerr,[18] also, has considered this in stating that he believed the difficulty of producing anaphylaxis with bacteria was in part due to the fact that their body substances were relatively poor in coagulable (antigenic) proteins. We have not been able as yet to study the matter extensively, but we have carried out a few experiments, as follows:

Typhoid bacilli from twenty-four agar cultures were weighed as a moist mass, ground up with salt, and then taken up in distilled water to isotonicity. After the addition of 0.2 cc. of $\frac{N}{1}$ sodium hydroxide to 100 cc. the suspension was heated to 60°C. for 30 minutes to prevent autolysis, and was then shaken for 4 to 5 hours with a motor, at room temperature. After filtration through a Berkefeld candle the clear solution gave a definite cloud after boiling and adding acetic acid. The filtrate was treated with heat and acid to remove coagulable protein. On the advice of Professor Gies the suspension was first brought to a boil and then small amounts of acid were added to prevent possible hydrolysis which might have occurred had the acid been added first. The filtrate from this was then half saturated with ammonium sulfate. Again a definite cloud was obtained, and when this was filtered clear, a second turbidity could be produced by complete saturation with the sulfate.

Although we have not yet obtained toxic reactions with these substances after isolation, perhaps because of the difficulty of obtaining them in sufficient amount, the presence of albumoses, substances which have often been found to possess primary toxicity for animals, suggests the possibility that their existence in the bacterial body might indeed contribute to the injury done by injections of bacteria. We found albumoses in extracts of typhoid bacilli not

[18] Doerr,[14] p. 1100.

only when the bacteria were grown on the ordinary peptone media but also on agar made without peptone to which nutrose or sodium caseinate has been added as an enriching substance.

Moreover, we have also had the experience with the typhoid bacillus which is referred to repeatedly by Vaughan when dealing with the colon bacillus; namely, that bacterial suspensions subjected to boiling are quite as toxic and often more so than are the unheated and living suspensions.

Thus, if three guinea pigs are injected with equal amounts of typhoid suspension, one with the living bacteria, the second with bacteria heated to 60°C. for 15 minutes, and the third with bacteria boiled for 5 minutes, the guinea pig receiving the boiled bacilli will often be the first one to grow sick and may die several hours before the others.

This may mean, of course, as Vaughan suggests, that the heated protein is more promptly split by the ferments of the body. It also suggests, however, that in addition to this the heating has left unchanged non-coagulable toxic constituents of the cell.

Passive Sensitization of Guinea Pigs to Bacteria.

Since in this work we were concerned primarily with the fundamental problems referred to in our introduction, we neglected for the present the purely technical difficulties connected with active sensitization and employed entirely the method of passive sensitization in which a more immediate and direct control over the relationship between the development of hypersensitiveness and the concentration of circulating antibodies could be exercised.

The experiments described in the following paragraphs illustrate the passive sensitization of guinea pigs by intraperitoneal injection with antityphoid serum. They show that unlike Kraus and his collaborators, we never succeeded in finding the animals sensitized in less than 3 to 5 days, the highest degree of sensitization being developed in about a week. This was true both when the whole animal was tested and when the reactions were obtained with the isolated uteri.

Passive Sensitization to Typhoid Protein. (*a*) *Experiment 1.*—Apr. 8, 1916. Guinea Pigs 1 to 5 inclusive are injected intraperitoneally with 1 cc. of Antityphoid Serum W which agglutinates 1: 10,000.

Apr. 10. Guinea Pig 5, weight 220 gm., is injected intravenously with 2 cc. of typhoid extract. No immediate symptoms.

Agglutination titer of this animal 1: 80++

1: 160+

Apr. 12. Guinea Pig 4, weight 190 gm., injected intravenously with 2 cc. of typhoid extract. Typical symptoms; falls to side; characteristic breathing; definite but not fatal shock. As well as control in 8 minutes. Control, weight 195 gm., shows no immediate symptoms.

Nos. 2 and 4 titrated on this day show agglutination up to 1: 160.

(*b*) *Experiment 2.*—Oct. 18, 1916. A series of guinea pigs, weight 220 to 255 gm., is intraperitoneally injected with 2 cc. of antityphoid rabbit serum with a titer of 1: 4,000.

Oct. 20. These guinea pigs show a titer of 1: 100 to 1: 200.

Oct. 23. Agglutinins in guinea pigs now about 1: 100.

Oct. 24. Titer diminishing to 1: 50 in most of them, others partially agglutinated 1: 100. On this day one guinea pig is injected intravenously with 1 cc. of typhoid extract. No sign of anaphylaxis. This animal, however, died over night, whereas the unsensitized control of the same weight remained alive.

Oct. 25. Typical shock obtained in two of the guinea pigs; no immediate symptoms in controls on injection of 1 cc. of extract.

It would not aid much in throwing light on the subject of our paper were we to multiply experiments like the foregoing. We may summarize by stating that, after injecting intraperitoneally antityphoid serum, and waiting a period of 3 to 5 days, at a time when agglutinins are still present to a considerable degree in the circulation of the guinea pigs, definite shock can be observed on injection of antigen. Earlier than this, however, in spite of a higher concentration of antibodies in the blood, no definite or severe acute shock could be elicited. It has seemed that to obtain very severe symptoms an interval of considerably longer than 1 day is necessary when an intraperitoneal first injection is practiced.

The following experiment, one of several similar ones, shows that when relatively large amounts of antigen are used the differences between normal and sensitized guinea pigs are essentially quantitative and not qualitative:

Experiment 3.—Jan. 16, 1917. Guinea Pigs 6 and 7 are intraperitoneally sensitized with 2 cc. of antityphoid serum.

Jan. 19. The following injections are made.

Guinea Pig 6, weight 170 gm., receives intravenously 5 cc. of typhoid extract. Immediate shock; death in 8 to 10 minutes. Control, weight 200 gm., receives 5.5 cc. of same antigen; quite sick, coughs, scratches nose, breathes irregularly. Gradual recovery.

Guinea Pig 7 receives 4 cc. of antigen. Immediate difficult breathing, coughing, very sick. Died after 3 to 4 hours. Control, same weight, receives 4 cc. of antigen. Difficult breathing. Same type of illness as No. 7 but less severe. Both controls dead next morning. No. 7 dies in afternoon.

Although it has been an irregular observation, the immediate development of anaphylactic symptoms in normal guinea pigs injected with large amounts of typhoid antigen has occurred with sufficient frequency to force itself upon our consideration. A typical instance of this is the following:

Experiment 4.—Feb. 20, 1917. A normal guinea pig, weight 175 gm., received 3 cc. of typhoid extract intravenously at 12 noon. Immediate coughing, respiratory distress, staggering. Temporary improvement; died at 4 p.m.

This observation we think has important theoretical bearing when correlated with later experiments on the isolated uterus. In this place we wish merely to record it for future reference. It is interesting to note in this connection that, although the sensitized animals show a much greater tendency to immediate reaction, unless the shock is fatal, they rapidly improve and approach the condition of slower intoxication, ordinarily found in the normal animals similarly treated. The following experiment illustrates these conditions:

Experiment 5.—Feb. 28, 1917. Guinea Pig 8, weight 155 gm., sensitized intraperitoneally with 2 cc. of antityphoid serum on Feb. 24, was injected intravenously with 2 cc. of typhoid extract. At the same time a control normal animal, weight 135 gm., was similarly injected. The sensitized animal showed immediate symptoms; coughing, difficult respiration. The control showed no immediate effects. After 10 minutes the sensitized animal had improved and both were alike and growing progressively sicker. Both died on the same afternoon.

Although the preceding experiments seem to furnish sufficient evidence that the dominating mechanism of acute shock in bacterial anaphylaxis is not one of intravascular antigen-antibody reaction, we nevertheless thought it wise to repeat the experiments of Kraus

and Amiradžibi who obtained shock, as stated before, when they injected simultaneously bacteria and antiserum. This we did for the sake of completeness although we feel sure that Kraus and Amiradžibi's results were referable to the intrinsic toxicity of their rabbit serum for guinea pigs.

In the experiment which follows, one of several similar ones, antigen injections were made into guinea pigs passively sensitized, into normal animals, and into normal ones that had received intravenously 0.5 cc. of antiserum about an hour before inoculation with the antigen. One example of such an experiment follows.

Experiment 6.—Apr. 9, 1917. Guinea pig, weight 199 gm., passively sensitized. Agglutination titer on Mar. 30, 1:80. 1.45 p.m. Receives intravenously 2.5 cc. of typhoid antigen. Very sick immediately; breathes with difficulty and irregularly for 5 minutes, then better. 9 p.m. Died.

10.45 a.m. Normal guinea pig, weight 200 gm., receives intravenously 0.5 cc. of typhoid antiserum. 1.55 p.m. Inoculated intravenously with 2.5 cc. of antigen. Not quite as sick as the preceding animal; illness develops more gradually but this animal does not recover from immediate symptoms as rapidly as the other. Dies next afternoon at 2.45 p.m.; *i.e.*, lives 6 hours longer than preceding guinea pig.

Normal guinea pig, weight 195 gm., inoculated with 2.5 cc. of antigen. 2.30 p.m. Very slightly sick, almost not at all; gradually sick. Very sick in p.m., but recovers and remains alive.

It would be of relatively little value for the purposes of this paper were we to protocol other experiments similar to or representing variations of the preceding. The results were by no means regular but, taken together, they pointed to certain fundamental conditions which are of considerable interest in connection with our later work.

We may summarize these facts by saying that: (1) Acute shock with convulsions and death is obtained more frequently and with lower dosage in sensitized than in normal guinea pigs. (2) Acute shock with death cannot be obtained with regularity in sensitized animals and is obtained in normal animals only on rare occasions when large doses are given. (3) To elicit acute shock with death even in sensitized animals requires at least a minimal fatal dose of the antigen and usually considerably more is required (two to three times) than the eventual killing dose of the antigen used. (4) Normal guinea pigs that are injected with a moderate dose of anti-

serum, followed within an hour by an injection of antigen, do not differ markedly from normal animals receiving the same amounts of antigen, in regard to acute symptoms. They have occasionally seemed sicker than the normal ones, but not with sufficient regularity to throw any light on any intravascular anaphylactic mechanism.

Analysis of Bacterial Anaphylaxis in Guinea Pigs.

We are confronted, therefore, with a problem which cannot be solved entirely by experiments on the living animal. On the one hand, our inability to obtain marked sensitiveness to relatively small doses, unless we allowed a considerable period to elapse between the administration of antiserum and the injection of antigen, points, as in serum anaphylaxis, to the importance of a cellular mechanism. On the other hand, the fact that with larger doses the differences between the sensitized and the normal become less well defined, though still apparent, would indicate that a preparation of the cellular elements by antibody absorption is not absolutely essential.

The alternatives are that either we are dealing with two reactions, one a purely intravascular one which is sufficiently powerful to cause acute shock with death only when very large amounts of antigen are used, and a cellular one which is in evidence only in sensitized animals, or that both in normal and in sensitized animals the mechanism is purely cellular and that the normal tissues of the guinea pig possess originally a slight degree of sensitiveness, analogous to normal antibodies in the circulation, which is merely increased by specific active or passive sensitization.

This problem can be further approached only by recourse to some of the physiological methods, such as heart and lung perfusion lately again applied to serum anaphylaxis by Manwaring, or the study of the isolated uterus—a method which has brought brilliant results in serum anaphylaxis in the hands of Dale[19] and Weil[20] and has lately been applied to the study of bacterial antibodies by Weil and

[19] Dale, H. H., *J. Pharm. and Exp. Therap.*, 1912–13, iv, 517.

[20] Weil, R., *J. Med. Research*, 1912–13, xxvii, 497; 1914, xxx, 317, 331; 1915, xxxii, 107.

Torrey[21] and by Manwaring and Kusama.[22] We accordingly have worked with all these methods but wish to report in this paper only on results obtained with the Dale method.

The first step in this work was to obtain typhoid anaphylactic reactions with the isolated uteri of animals intraperitoneally injected with antityphoid serum. Here again, we found that the interval after injection was much longer than we expected and uteri from these guinea pigs would often react only after 3, 4, and 5 days, several of those tested after 1, 2, and 3 days failing to react. The highest degree of sensitiveness is not acquired after injections of 2 cc. of serum (titer from 1:4,000 to 1:10,000) until about the 5th to the 8th day after injections, a time at which the agglutinins in the circulation of the animal are beginning to diminish. This point of the relation between the circulating agglutinins and the sensitiveness of the whole animal is one of great importance which we believe marks an essential difference between the study of the isolated uterus and the whole animal.

Fig. 1 represents the reaction of a passively sensitized uterus to typhoid antigen when the typhoid bacilli were ground in salt and were extracted with very slightly alkaline salt solution. The bath capacity in these earlier experiments was 200 cc.; in later ones this was reduced to 50 cc. for purposes of economy.

Fig. 2 represents a similar reaction of a highly sensitized uterus where it is shown that the sudden instillation of large amounts of typhoid antigen may give rise to a repetition of spasm, three or four separate shocks being noted. This has not often been the case in our experiments, the uterus usually going into a prolonged single spasm, but was interesting to us because it was a graphic representation of a repetition of shock which we once noticed in young goats on the reinjection of typhoid bacilli. It is also worth noticing that occasionally when a uterus has begun to relax, if more antigen is added, another shock can often be induced even though the first instillation seems to have contained much more antigen than could possibly be absorbed within the uterine cells, a fact which points to the importance of concentration rather than actual amount,

[21] Weil, R., and Torrey, J. C., *J. Exp. Med.*, 1916, xxiii, 1.

[22] Manwaring, W. H., and Kusama, Y., *J. Immunol.*, 1917, ii, 157.

since it is analogous to observations made by pharmacologists in similar experiments with drugs in which the essential element of action seems to depend upon concentration and not upon actual quantity. Repeated addition of the antigen, however, leads to rapidly diminishing intensity of shock and desensitization. Fig. 2 also incidentally seems to show that even when the surrounding antigen is removed, the stimuli which lead to spasm may, on occasion, continue, pointing to the essentially intracellular occurrence of the reaction between antigen and antibody which is the ultimate cause. We should emphasize, however, that the above is the only instance in which such repeated and strong reactions were obtained in this way.

Having thus ascertained that passive sensitization of the animal is easily determined by the Dale reaction, we thought that it would be easy by the same method to determine whether the shock elicited in normal animals by large doses of antigen depended merely upon a lesser degree of cellular sensitization. Were this the case, large amounts of antigen added to a bath containing a normal uterus should induce at least some degree of reaction. Figs. 3 and 4 represent these experiments.

It is apparent from both these charts that large amounts of antigen exert no effect upon the isolated normal uterus. From this it would seem clear that, whatever is the mechanism of injury which induces response of the whole animal to injections of typhoid antigen, it is not one in which cellular sensitiveness is involved, at least, in an anaphylactic sense. This observation we consider of great importance, for it indicates that to the mechanism of bacterial injury in the normal animal, there is superadded another and cellular one in the sensitized animal. It might, of course, be considered that cells other than uterine might be sensitive to direct reaction with the bacterial products in the normal guinea pig. But Weil, especially, has shown a remarkable parallelism between hypersusceptibility in general and uterine reactions in guinea pigs, and in the light of his work and that of others it would seem most likely that the normal animal lacks cellular sensitiveness to the unchanged bacterial substance and that the mechanism of injury, therefore, must be regarded as in large part an intravascular one until the animal is sensitized.

Incidentally, we have shown that with bacterial anaphylaxis the same phenomenon is present that was found in Dale's experiments with horse serum anaphylaxis; namely, that the normal uterus cannot be sensitized, even by prolonged soaking in immune serum, and the union with the antibodies in serum is one in which the antibodies become an intrinsic part of the cells and are not merely physically fixed to the cell exterior.

Up to this point we have worked with bacterial extracts and have found that, in fundamental principles, reactions obtained with bacterial antigen are essentially identical with those obtained with such antigens as horse serum, etc. Inasmuch as the bacteria in infectious diseases are present in the body primarily as living and growing cells, it would seem important to ascertain whether sensitive cells can react to the whole bacterial body, or whether, as we assumed above, there must be a preliminary extraction or solution of the bacteria before injury can be done to the tissue elements. In consequence, we carried out a number of experiments in which heavy suspensions of living bacilli were added to Locke's solution in which was suspended a uterus from a sensitized animal. The opposite horn was, of course, always tested for sensitiveness with typhoid extract. Figs. 5 and 6 illustrate these experiments and show that the whole bacilli exert little or no effect on the sensitized organ.

We must assume, therefore, that where bacteria are injected into the sensitized animal, or are invading the living animal or human body, injury to sensitized tissues must be preceded by a liberation of antigens. Does this occur in the blood stream by lysis? One way of determining this would be to add the serum of a guinea pig injected with whole typhoid bacilli some time before, into a bath containing a sensitized uterus. We tried this, but obtained confusing results owing to the fact that normal guinea pig serum in itself exerts irritating effects upon the isolated guinea pig uterus. In consequence, we resorted to precipitin and complement fixation reactions. The former were not satisfactory, but the latter gave us, we believe, a clear indication of the presence of dissolved antigen in the circulation of guinea pigs intravenously injected an hour previously with whole typhoid bacilli. Such an experiment follows.

Experiment 7.—Guinea pig, weight 300 gm., received five slants of living whole bacilli, washed once in salt solution, intravenously. After 1 hour the animal was bled and cultures were taken on agar plates from its whole blood and from the serum after clotting of the blood. The following experiment was then done with the inactivated serum of this animal as antigen; a strong antiserum as antibody; and a fresh guinea pig complement previously titrated carefully against the hemolytic system.

Tube.	Two units of complement added to the following.	Inhibition of hemolysis.
1	0.1 cc. of typhoid antiserum +1.5 cc. of salt solution	0
2	0.3 " " serum of injected guinea pig +1.2 cc. of salt solution	0
3	0.1 " " typhoid antiserum +0.1 cc. of serum of injected guinea pig +1.4 cc. of salt solution	+++
4	0.1 cc. of typhoid antiserum +0.2 cc. of serum of injected guinea pig +1.3 cc. of salt solution	++++
5	0.1 cc. of typhoid antiserum +0.3 cc. of serum of injected guinea pig +1.4 cc. of salt solution	++++
6	1.6 cc. of salt solution	0

To these tubes we added two units of complement and, after 1 hour in the water bath, sensitized red cells were added. The results obtained are expressed in terms of Wassermann reactions as read in 1 hour in the water bath and over night in the ice chest, this being necessary because 0.3 cc. of the injected guinea pig serum was slightly anticomplementary and absolutely complete reactions could not be read until the next morning in this tube and in Tube 5.

There is one possible error which, however, we think we can discount; namely, the culture taken from the whole guinea pig blood in this and other experiments always yielded considerable numbers of colonies of typhoid bacilli, as many as 1,000 or so, which inclines one to consider the possibility that the complement fixation might have been due to whole bacilli. However, the serum from such blood was entirely clear and was further centrifugalized before use, and cultures taken from the serum in this experiment before it was heated for inactivation yielded only two to three colonies per 0.2 cc., which indicated to us that, as one would expect, the great bulk of typhoid bacilli were caught and held in the clot. Had the clot been allowed to stand in the ice chest over night for the expression of the sera, it might still have been possible that an extraction of these typhoid bacilli might have taken place, but we centrifugalized the blood as

soon as clotted and extraction therefore seems unlikely. We believe, therefore, that these experiments indicate that in the circulation of the guinea pig injected with live typhoid bacilli, and presumably, therefore, in the spontaneously infected body, antigen is in some way liberated to the circulating blood, which then could react with sensitized tissue as in our uterus experiment.

If this is the case, the next link in our chain would be to determine whether we could also trace the actual formation of toxic products, such as Friedberger's anaphylatoxin, or Vaughan's split products, or the substances we have spoken of as proteotoxins in the circulating blood. We have attempted to do this in two ways. One consisted in repeated attempts to produce acute symptoms of shock in guinea pigs by injecting intravenously blood serum taken from normal animals rendered severely sick both by typhoid bacilli and typhoid extract. We have never succeeded in this. Next we attempted to produce contraction in the normal guinea pig uterus, by adding to the bath of Locke's solution such guinea pig serum. In this we did not succeed, partly because it was never possible to add large amounts of guinea pig serum to these baths without running the risk of obtaining the contraction incident to the instillation of normal guinea pig serum. When we did so, no marked contrast appeared between the result of putting in normal guinea pig serum and putting in the same amount of the serum of an animal dying of typhoid bacilli (Fig. 7).

We cannot, therefore, with any of the methods now at our disposal, demonstrate the formation in the blood stream of the typhoid-infected animal of the poisons which are responsible for acute anaphylactic shock. Does this negative the intravascular production of such toxic substances? We think not.

In the first place, it must be remembered that, whatever may be the mechanism of injury going on in an animal infected and poisoned with typhoid or other bacilli, the process is a gradual one extending over a period of from 4 to 12 hours or longer, even when large doses are given. It is likely that the poisons which are formed are absorbed with considerable speed, this being responsible for the symptoms developing in the animal. It is not likely, therefore, that we could find at any one time a sufficient amount of the toxic substance present in the limited quantity of blood serum which we can readily inject into

an animal or instil into the Dale's bath, to expect to obtain acute reactions. For with bacterial anaphylaxis especially, it must be remembered that acute shock is obtained with no great regularity and only in animals highly sensitized when considerable quantities of bacterial antigen are administered.

Figs. 8 and 9 demonstrate incidentally that the conditions observed with bacterial antigens are to a great extent similar to those observed when other cellular antigens, such as erythrocytes, are used. Fig. 8 shows a typical reaction with the uterus of a guinea pig which had received 2 cc. of amboceptor intraperitoneally 3 days before, when hemolyzed red cells were added to the bath, and also indicates subsequent desensitization; and Fig. 9 shows the complete failure of reaction when the cells are added as "whole" cells unhemolyzed to a similarly sensitized organ. Reaction can be elicited by cells hemolyzed with distilled water or hemolyzed directly in the Locke's bath by the addition of amboceptor and complement, thus showing the preliminary function of solution of the cells in the blood stream carried out by the circulating antibodies. We do not insert a chart to show this because these records are very long owing to the necessity of allowing plenty of time between the instillation of amboceptor, complement, and red cells, to eliminate non-specific irritation of the uterus by these substances separately. The results were clear-cut, contraction usually beginning at about the time when hemolysis began and progressively increasing towards its completion.

Another point which formed an important part of our scheme of work was the study of the possible influence of circulating antibodies upon the reaction between antigen and sensitized tissues. To obtain light on this subject, we proceeded by the following two methods. In one series of experiments antiserum was injected into sensitized animals just before the injection of antigen, in order to ascertain whether by this means shock was diminished or eliminated. In another series, antigen was incubated for from 1 to 3 hours with antiserum and the mixture instilled into Locke's baths containing sensitized uteri.

An experiment by the former manner of procedure is as follows:

Experiment 8.—Apr. 7, 1917. 2.30 p.m. Passively sensitized guinea pig, weight 190 gm., receives intravenously 0.5 cc. of strong typhoid antiserum. After 14 minutes receives 3 cc. of typhoid antigen. Very slight symptoms; breathing slightly labored but runs about immediately and shows hardly any illness for the first 10 or 15 minutes.

Passively sensitized guinea pig of the same lot as the preceding animal. Agglutinin titer about 1:160. Receives 3 cc. of typhoid antigen. Very sick immediately; coughs and breathes irregularly. Much sicker than the preceding guinea pig.

These two animals represent a few of a considerable number similarly treated. With a few exceptions it seemed fairly definite that the presence of a considerable concentration of antibodies in the circulation delayed rather than hastened the development of acute symptoms.

The explanation for this we think is to be found more definitely in the following experiments with isolated uteri.

Fig. 10 represents an experiment in which the typhoid antigen which in substance consisted of a turbid extract containing not only extracted dissolved substances, but also particles, was exposed to the action of antiserum until a heavy precipitate had formed. This precipitate was then washed and added to the Locke's bath. No reaction resulted. The clear supernatant fluid, however, gave a sharp reaction in all cases, whether or not we added an excess of antiserum and reincubated.

Inasmuch as our antiserum gave only a slight precipitation with clear, filtered typhoid extract, it remained uncertain whether or not the experiment represented by Fig. 10 meant only that undissolved particles had been precipitated or whether it also signified that a part of the dissolved antigen was rendered useless by the antibodies. After several unsuccessful attempts to obtain a clear answer to this query by working with typhoid antigen and antibody, we decided to repeat the experiment using human serum as antigen and antihuman rabbit serum as antibody. Fig. 11 represents such an experiment.

Here 0.5 cc. of human serum in 1 cc. of salt solution was incubated with 0.3 cc. of a strong antihuman rabbit serum. A heavy precipitate formed which was thrown down in the centrifuge and washed once with salt solution, then shaken up in a small amount of salt solution and added to the bath containing a passively sensitized uterus. No

TABLE I.

Date.	Antigen.	Normal guinea pigs.		Sensitized guinea pigs.	
		Weight.	Remarks.	Weight.	Remarks.
1916	*cc.*	*gm.*		*gm.*	
Mar. 31	3	240	Lives.	250	Sensitized Mar. 27. No immediate symptoms; dead next day.
May 5	2	227	Dead next a.m.	215	Immediate.
		205	Dead 5 to 6 hrs.	215	Sensitized Mar. 31. Symptoms marked; dead next a.m.
Apr. 13	2	195	Sick but no shock; died next a.m.	190	Sensitized on Apr. 8. Definite shock; dead same afternoon.
	1	180	No symptoms; lives.	180	Mild shock; dead same afternoon. Agglutinations of guinea pigs of same series up to 1 : 160.
Apr. 14	3 (ureter).	180	Immediate shock-like symptoms; dead next a.m.	180	Sensitized Apr. 8. Typical shock; dead next a.m. Sensitized sick more rapidly; mate agglutinin, 1 : 320.
Apr. 15	3	190	No shock; dead next a.m.	180	No shock; lives. Mate agglutinin, up to 1 : 80.
Oct. 24	1 (strong).	220	Slight symptoms; lives.	220	Sensitized Oct. 28. No shock; dead next a.m. Mate agglutinin, up to 1 : 100.
1917					
Jan. 19	4	*	Immediately sick; died next a.m.	*	Sensitized Jan. 1. Heavier than control. Shock; died in 4 hrs. Mate agglutinin, 1 : 100.
	5	200	Distinct moderate shock; died next a.m.	170	5 cc. Acute shock; died.
Apr. 4	25	300	No symptoms; died next a.m.	270	Severe shock; lives. Agglutinin, 1 : 80.

*Error in records; no note of weights made.

reaction occurred. However, again the addition of the supernatant fluid to the same bath resulted in a strong reaction.

This experiment was in entire harmony with those performed with typhoid antigen and antibody. It showed that antigen precipitated by antibody was thereby neutralized as far as its powers to react with sensitized tissues were concerned. It also showed, incidentally, a fact immunologists have known for some time, that it is extremely difficult to remove all antigen from solution by precipitation with antibody, and that most of the substance of a specific precipitate is derived from the antiserum.

We still wished to determine if possible, whether an animal sensitized to typhoid protein was or was not thereby rendered more vulnerable; *i.e.*, more easily killed by typhoid antigen. Table I, constructed from scattered observations made from time to time throughout our work gives some clue to the question, though it by no means settles the matter conclusively.

It will be seen that as a rule the sensitized animals died more quickly that did the normal controls, in spite of the fact that they were slightly protected by the presence of small amounts of antibody. This point will need further experimentation, which is already in progress.

SUMMARY.

We have attempted in the preceding experiments the beginning of an analysis of bacterial anaphylaxis and its relation to the occurrences in the animal body during an infectious disease. We have shown that the sensitization of the tissues of guinea pigs, as indicated by the isolated uterus, required 3 to 5 days even when passive sensitization was employed, and that in these relations conditions with bacterial sensitization were entirely analogous to those revealed for serum anaphylaxis by Dale and Weil especially. It has become apparent that the sensitized uterus reacted not at all with whole bacteria or whole red cells, or, in other words, that before reaction with sensitized organs could occur an extraction or solution of the bacterial cell must take place. That bacteria yield some of their substance to the circulating blood during the course of infection was to be expected, but it has been definitely indicated, we think, by our complement fixations.

The mechanism of injury in the sensitized animal or in the human being so far along in typhoid fever that antibodies have begun to develop is in part one in which antigen, derived from the bacilli and brought into solution, or rather suspension, in the blood stream, reacts with antibodies which are from the beginning, or have subsequently become, integral parts of the cell protoplasm, the entire process taking place within the cell. This last point is indicated by the failure to sensitize by simply soaking the normal uterus in antiserum.

This, however, cannot be the entire story of injury. We know that typhoid antigen injected into normal animals in moderate amounts will render them gradually sick and eventually kill them. Also, a sufficient amount injected into a normal animal will occasionally produce acute symptoms, in every respect similar to the reaction produced in sensitized animals by smaller doses. We have shown that such acute symptoms in normal animals were not due in any degree to tissue sensitiveness, since even very large quantities of antigen will produce no response on the part of the normal uterus. It is reasonable to suppose, therefore, that the injury, gradual or acute, in the normal animal, is in no respect referable to tissue sensitiveness to the whole antigen, but rather must be referred to some series of phenomena which occur in the circulation. The acute shock of normal animals may possibly, therefore, be entirely due to an intravascular reaction. Whether this is one of antigen-splitting, or of antienzyme removal in the sense of Jobling is a point on which these experiments throw no light.

It is true that we have never succeeded in producing acute toxic symptoms either in the whole animal or in the isolated uterus with serum from animals acutely ill. This we eliminate as negative evidence inasmuch as we believe that the toxic substances need at no given time be present in the blood stream in sufficient concentration to render such an experiment successful. They are probably absorbed and do their injury almost as rapidly as formed, an assumption which is based on the speed with which symptoms develop.

It is possible, and not to be denied on the basis of any experiment that we can devise at present, that the gradual illness of the normal animal and the occasional acute shock of these animals may be based on entirely different mechanisms. In both cases, however,

in normal animals, they seem to be intravascular. And since the symptoms of acute shock which can be produced in sensitized animals with moderate doses can also, though only occasionally, be produced in normal animals with larger doses, it is reasonable to suppose that the poisons produced intracellularly in the one may be similar to those produced intravascularly in the other.

It does not seem likely that the specific circulating antibodies are in any way sources of increased injury to an animal spontaneously infected with bacteria. If sufficiently powerful at the beginning they may even prevent tissue injury, first by increasing phagocytosis, then by producing intravascular agglutination, and finally, as indicated by our experiments, even by removing a part of the antigen from possible reaction with the cell, though in this last respect our experiments indicate that they functionate imperfectly. It is more probable that their chief protective action to the sensitized body lies in removing the whole bacteria from the possibility of intravascular disintegration, which, as we have shown, is prerequisite to anaphylactic injury of the tissues of the host.

We would tentatively summarize our opinion as to the occurrences in the typhoid-infected body as follows: Early injury is probably due to disintegration of part of the bacteria in the course of which albumose-like bodies are liberated, and, following which, intravascular reactions result in the formation of toxic substances, perhaps by some form of proteolysis.

Since the accumulation of bacteria during these stages is relatively slight, this form of injury probably plays little part in producing symptoms. Indeed, the experiment by which acute injury is produced in the normal guinea pig by the sudden injection of several times the lethal dose of partly dissolved bacteria, finds no analogy in the spontaneously diseased body.

At this time the tissues are not sensitive, but as antigen absorption progresses and the tissues are stimulated to react, sensitiveness develops, which renders them much more delicately amenable to injury by direct reaction with even small amounts of dissolved but otherwise unaltered antigen. This process is directly counteracted by circulating antibodies which tend to remove the bacteria from the possibility of yielding their antigen to solution by agglutinating

them, aiding phagocytosis, and to a slight extent even neutralizing dissolved antigen.

It seems likely, therefore, that the symptoms which appear as the incubation time ends are largely those due to cellular sensitization which probably begins before any considerable amount of circulatory antibodies is present. The circulating antibodies would seem to have little or nothing to do with intravascular injury, the ferments responsible for this, however much it may occur, probably consisting of the non-specific proteases studied in this connection by Jobling.

Finally it appears that highly sensitized animals are more easily killed by typhoid antigen than are normal animals, provided they do not dispose over unusually large amounts of circulating antibodies.

Cure would consist of a gradual checking of growth and final destruction of the bacteria, and the consequent cessation of antigen liberation, but delicate hypersusceptibility would probably persist for some time after cure and immunity have been established. Just what the relation between tissue hypersusceptibility and immunity is remains a problem for further study.

EXPLANATION OF PLATES.

PLATE 38.

FIG. 1. Typical reaction of sensitized uterus to typhoid antigen.

FIG. 2. Repeated spasm on inoculation of large amounts of antigen. A reaction like this was obtained very exceptionally. No other was obtained in which the same degree of repetition was noticed.

FIG. 3. Failure of normal uterus to react to typhoid antigen. This uterus had been soaked in immune serum for 3½ hours.

FIG. 4. Failure of normal uterus to react to typhoid extract in spite of the repeated inoculation of large amounts. The amounts instilled in the bath in this case were at least four times greater than sufficient to kill a guinea pig of the same size.

FIG. 5. Failure of sensitized uterus to react to large doses of living typhoid bacilli.

PLATE 39.

FIG. 6. Another instance of the failure of living typhoid bacilli in large amounts to stimulate sensitized uterus.

FIG. 7. Failure of normal uterus to react when the serum of a guinea pig dying of living typhoid bacilli was added. Attempt to demonstrate proteotoxin in serum of a typhoid-infected animal.

FIG. 8. Typical reaction of the uterus of a guinea pig passively sensitized with amboceptor when hemolyzed red cells are added.

FIG. 9. Uterus of a guinea pig passively sensitized to red cells. The chart shows the failure of reaction when cells are intact and reaction when cells are disintegrated by hemolysis.

FIG. 10. Uterus passively sensitized to typhoid antigen. The chart shows the failure to react when precipitate produced by the action of antiserum is added. Reaction when supernatant fluid of such a mixture is added.

FIG. 11. Uterus passively sensitized to human serum. Failure of reaction when precipitate produced by reaction of human serum and antihuman serum is added, but prompt reaction when the supernatant fluid of such a precipitin reaction is instilled into the bath.

FIG. 1.

FIG. 2.

FIG. 5.

FIG. 4.

FIG. 3.

(Zinsser and Parker: Bacterial anaphylaxis and infection.)

FIG. 7.

FIG. 6.

FIG. 9.

FIG. 8.

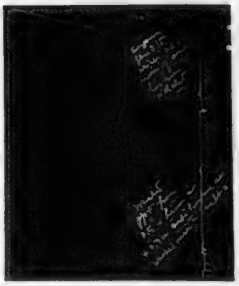


FIG. 11.

FIG. 10.

(Zinsser and Parker: Bacterial anaphylaxis and infection.)

INCREASED VIRULENCE OF THE HOG-CHOLERA BACILLUS PRODUCED BY PASSAGE THROUGH RABBITS.

By CARL TENBROECK, M.D.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

(Received for publication, May 18, 1917.)

In a previous paper (1) the writer mentioned the attempt to modify the carbohydrate reactions of a culture of the hog-cholera bacillus by passing it through a series of rabbits. The only change noted was an increase in virulence, and it is the object of this paper to record this change.

Since the time of Pasteur many organisms have had their virulence increased by animal passages but the recorded results of the passage of the hog-cholera bacillus through rabbits are somewhat conflicting. Moore (2) passed a typical organism through a series of twenty-six rabbits and concluded that there was no increase in virulence for this animal. He judged the virulence by the time taken to kill the animal and did not consider the amount of culture necessary to produce death. Previous to this Selander (3) had reported the rapid increase in virulence of a hog-cholera bacillus by rabbit passages and his work was apparently confirmed by Metchnikoff (4). Smith and Moore (5) had shown, however, that Metchnikoff was working with the swine-plague bacillus and as Moore could not confirm the work of Selander he concluded that the latter was probably also working with the swine-plague organism.

Smith (6) later worked with a culture of the hog-cholera bacillus of low initial virulence further attenuated by age, which would not kill rabbits when injected under the skin. By animal passages, he increased the virulence to the point where subcutaneous injections were fatal.

Later Smith and Reagh (7) in attempting to modify the agglutinability of a strain of the hog-cholera bacillus, passed it through a series of fourteen rabbits. As a result of this passage the virulence was increased from a minimal fatal dose of 0.1 cc. in the stock strain to 0.00001 cc. in the passage strain. It has already been pointed out (1) that this increased virulence still persists to a certain degree after a lapse of 15 years.

The organism with which we worked is a stock culture known as Hog-cholera XII. It was isolated by Dr. Smith in 1914 from the spleen of a pig dying from hog-cholera. Soon after this it was passed through a rabbit and since that time it has been kept on slant agar in the cold, transfers being made monthly.

Culturally it is a motile, Gram-negative rod, growing readily on the ordinary media and forming acid and gas in dextrose bouillon but not attacking lactose or saccharose. It is quantitatively agglutinated by serum from rabbits injected with other strains of the hog-cholera bacillus and when injected into animals it causes the production of agglutinins for other strains of the hog-cholera bacillus.

Starting in January 1916 this culture was passed through a series of eleven rabbits; the essential details of this passage will be found in Table I. The passage was made directly from one animal to the next by using a suspension of crushed spleen for the inoculation. After the third transfer the inoculation was made by rubbing a small amount of the spleen suspension into the shaven skin of the next rabbit.

TABLE I.

Passage of Hog-Cholera XII through Rabbits.

Passage.	Weight of rabbit.	Method of infection.	Material and dose.	Length of life.	Remarks.
	gm.			*days*	
1	1,199	Subcutaneous.	0.5 cc. of 24 hr. bouillon culture.	6	Typical lesions.
2	1,172	"	Spleen suspension.	6	" "
3	1,274	"	" "	7	" "
4	1,305	Cutaneous.	" "	7	" "
5	1,430	"	" "	7	" "
6	2,073	"	" "	9	" "
7	2,089	"	" "	10	" "
8	2,273	"	" "	8	" "
9	2,029	"	" "	7	" "
10	2,308	Subcutaneous.	0.000001 cc. of 24 hr. bouillon culture from Rabbit 9.	9	" "
11	2,336	"	0.00000001 cc. of 24 hr. bouillon culture from Rabbit 10.	6	" "

It would be difficult to estimate accurately the relative virulence of this culture from the duration of the disease in the various animals but a comparison of the number of organisms necessary to kill gives us very definite results. With the stock culture one must use somewhat over 0.00001 cc. of a 24 hour bouillon culture to produce death.

Of four rabbits given a subcutaneous injection of this amount one died in 12 days, one showed an increased temperature and loss in weight, the other two showed no effects. One rabbit that was given 0.001 cc. subcutaneously died in 4 days. With the strain of the same bacillus passed through the rabbits, 0.00000001 cc. of a 24 hour bouillon culture injected subcutaneously into a rabbit weighing 2 kilos causes death in about 6 days. The passage strain has therefore been increased in virulence about one thousand times and plate counts show that the number of organisms necessary to infect has been reduced from approximately 20,000 to 20.

The type of disease produced by the more virulent organism shows no striking departure from that caused by the original culture. This organism seems to have a greater power of penetration than do most cultures of the hog-cholera bacillus, as it causes only a slight local lesion. The bacteria apparently enter the body through the lymphatics, for the axillary and inguinal lymph nodes on the side of the inoculation are enlarged and congested and often show large areas of necrosis.

The passage through rabbits has produced no change in the morphology of the organism that can be detected either in films made from the spleen of an animal dying from infection or in films made from cultures. The passage strain is slightly more motile than the original culture but this difference is not marked. It is also more susceptible to agglutinins, in that clumps are formed in a shorter time, but the readings after 2 hours' incubation are the same. The passage of the original strain through one rabbit makes it as susceptible to agglutinins as is the strain passed through eleven rabbits. With some immune sera the parent strain is agglutinated in slightly higher dilutions than is the passage strain but here too the difference is not great.

SUMMARY AND CONCLUSION.

By passage through a series of eleven rabbits a culture of the hog-cholera bacillus has increased its virulence a thousand times. A subcutaneous injection of twenty organisms, or 0.00000001 cc. of a 24 hour bouillon culture, or a drop of a bouillon culture rubbed lightly into the shaven skin, produces, in the rabbit, a characteristic disease resulting in death on or about the 6th day.

BIBLIOGRAPHY.

1. TenBroeck, C., *J. Exp. Med.*, 1916, xxiv, 213.
2. Moore, V. A., *U. S. Dept. Agric., Bureau Animal Industry, Bull. 6*, 1894, 97.
3. Selander, *Ann. Inst. Pasteur*, 1890, iv, 545.
4. Metchnikoff, E., *Ann. Inst. Pasteur*, 1892, vi, 289.
5. Smith, T., and Moore, V. A., *U. S. Dept. Agric., Bureau Animal Industry, Bull. 6*, 1894, 60.
6. Smith, T., *U. S. Dept. Agric., Ann. Rep., Bureau Animal Industry*, 1895–96, xii and xiii, 161.
7. Smith, T., and Reagh, A. L., *J. Med. Research*, 1903, ix, 270.

THE SIGNIFICANCE OF AGGLUTININS IN THE IMMUNITY OF THE RABBIT TO THE HOG-CHOLERA BACILLUS.

By CARL TENBROECK, M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Received for publication, May 18, 1917.)

A great deal of experimental work has been done on agglutinins, yet we have very little evidence as to the part they play in immunity to any given infectious disease. Much of the work tends to show that they are not indicators of immunity but in spite of this the agglutinin titer is often used as an index of immunity after antityphoid vaccination and when the titer falls it is regarded as an indication for revaccination. In the production of various immune sera it is often assumed that the height of the agglutination titer is an index of the antibody content but the evidence on which this assumption is based is not clear. We have endeavored to throw some light on the relation of agglutinins to immunity by the use of the highly virulent culture of the hog-cholera bacillus described in the previous paper (1).

A large part of the experimental work on agglutinins has been done with the rabbit and the horse inoculated with the typhoid bacillus, and the great weakness of this work is that in the former animal at least, this organism does not produce a true invasive disease but acts only when large numbers of bacteria are given. On the other hand, the hog-cholera bacillus produces, in the rabbit, a true disease resembling in many respects typhoid fever in man.

HISTORICAL.

In 1894 Smith and Moore (2) showed that heated cultures of the hog-cholera bacillus would not produce an immunity in the rabbit but that the injection of a living attenuated culture would produce an active immunity to the more virulent organism. They did not at this time study the agglutinins, but later Smith and Reagh (3) produced agglutinins by the injection of heated as well as living cultures of the hog-cholera bacillus.

Shoukévitch (4) reports work similar to that to follow in which, using the hog-cholera bacillus and the rabbit, he showed that the injection of heated cultures caused an increase in the complement-fixing bodies, agglutinins, and opsonins but that this was not associated with an immunity. The injection of a living culture of low virulence caused a very slight production of these bodies but did cause an active immunity to more virulent organisms. Of fifteen animals tested in this way, five showed no effects, one died with a typical septicemia, two died from acute intoxication, and seven died in from 15 to 70 days with a paralysis of various groups of muscles. All showed little or no increase in the immune bodies following the injection of the culture of low virulence.

Whether the phenomenon described by Bull (5) under the name of *intra vitam* agglutination is related to *in vitro* agglutination is a question that will not be considered in this paper but we will consider the relation of this phenomenon to immunity to the hog-cholera bacillus. Bull has recorded experiments with a rather large series of organisms, the avirulent members of which are clumped when injected intravenously into normal rabbits, while the virulent members are not clumped and are found in the circulation for some time after the injection. When the bacilli are clumped they rapidly disappear from the blood stream and in a half hour after the injection, films from the liver and lungs show many polymorphonuclear leukocytes filled with organisms. Virulent organisms are not clumped in the circulation of normal rabbits but are rapidly clumped and phagocyted when injected into immune animals. Bull says (5) "The degree of agglutination and opsonization of bacteria within the animal body is inversely parallel to the infectiousness of the bacteria for the host" but he is careful to state that exceptions may be found to this rule.

EXPERIMENTAL.

Cultures Used.—Reference has already been made to the highly virulent test culture (1). The parent culture that had not been passed through rabbits and having the laboratory number XII was used as a vaccine and in small doses of the living culture to produce an immunity.

Hog-cholera Neb., isolated by Dr. Smith in 1886 (6), is now a culture of very low virulence. It is a typical hog-cholera bacillus except for its lessened virulence.

Hog-cholera Ark., isolated by Dr. Dinwiddie in 1889, was referred to by Dr. Smith in 1903 (3) and the cultural characters have been reported more recently (7). It is an organism of moderate virulence, probably due in part at least to its prolonged cultivation on artificial media.

Use of Heated Cultures.

The first experiment was made with a vaccine prepared by suspending the 24 hour growth from an agar slant inoculated with Hog-cholera XII in 10 cc. of salt solution and heating for 1 hour at 60°C. The heated suspension was incubated over night and several loops were transferred to bouillon in order to be sure that it was sterile. Having proven that all the bacteria had been killed the vaccine was injected subcutaneously into two rabbits of approximately equal weight. The first injection was of 0.5 cc. and was followed in 5 days by 1 cc. 6 days later they were each given 2 cc. Both rabbits bore the injections well, showing only a slight and temporary loss in weight.

18 days after the beginning of the treatment and 7 days after the last inoculation these two rabbits, together with a control, were bled from the ear vein and the serum of the inoculated animals was tested for agglutinins against the strain of the hog-cholera bacillus with which they had been injected and also against the strain that had been passed serially through rabbits and with which they were to be inoculated in order to test their immunity.

In order that the rabbits might have every opportunity to show any immunity they might have gained by the injection of the heated cultures they were tested by the cutaneous method, two drops of a 24 hour bouillon culture being lightly rubbed into the shaven skin of each animal. This test was made 2 days after the bleeding from the ear vein and 9 days after the last injection of the heated cultures. The results of this experiment are summarized in Table I.

TABLE I.

Test of the Power of Heated Cultures to Produce Agglutinins and Immunity.

Rabbit No.	Vaccinated.	Limit of agglutination* for Hog-cholera XII.		Weight.	Immunity test. Result of cutaneous inoculation with test culture.
		Stock.	Passage strain.		
				gm.	
1	Three times with heated culture.	$\frac{1}{12,800}$	$\frac{1}{12,800}$	1,823	Died in 5 days.
2	Same as No. 1.	$\frac{1}{12,800}$	$\frac{1}{12,800}$	1,889	" " 7 "
3	Control.	Not tested.		2,006	" " 6 "

* The limit of agglutination is the highest dilution in which clumps of bacteria can be seen with the naked eye. A 24 hour bouillon culture was used as an antigen throughout.

It will be seen that while both treated rabbits had in their serum agglutinins that could be demonstrated in a dilution of $\frac{1}{12,800}$ they

had no immunity. Both died in approximately the same number of days after the inoculation as did the control. At autopsy all three rabbits showed the same local as well as visceral lesions, the only essential difference being that both vaccinated rabbits had a small area of pneumonia. It is quite possible, however, that the latter was due to another organism that was affecting our stock rabbits.

A similar experiment, but with results that are not so clear-cut, was made some time later. The preparation of the vaccine and the dosage was the same as in the preceding experiment except that the culture used was the highly virulent rabbit passage strain. The test for immunity was made by injecting subcutaneously 0.0000001 cc. of a 24 hour bouillon culture of the passage strain. The results are summarized in Table II and show that of the vaccinated rabbits the one that had the higher agglutination titer died, while the other one survived without showing any marked effects from the inoculation. Examination of the controls shows that we were using about the smallest dose that would cause an infection, so that the apparent immunity of the one vaccinated rabbit might be due to causes other than the injected vaccine such as a natural immunity or too small an infecting dose. In spite of this discordant result it is clear that there was no demonstrable immunity in the rabbit with the higher agglutination titer.

TABLE II.

Test of the Power of Heated Cultures to Produce Agglutinins and Immunity.

Rabbit No.	Vaccinated.	Limit of agglutination for test culture.	Weight.	Subcutaneous injection of test culture.	
				Amount.	Result.
			gm.	*cc.*	
4	Three times with heated culture.	$\frac{1}{25,600}$	2,011	0.0000001	Died in 9 days.
5	Same as No. 4.	$\frac{1}{12,800}$	2,030	0.0000001	No effect.
6	Control.	0	2,683	0.0000001	Died in 9 days.
7	"	0	1,763	0.00000001	No effect.

Use of Living Cultures to Produce Immunity.

The immunization of rabbits by the injection of living cultures of the hog-cholera bacillus is very difficult, for their resistance may be overcome by the injection of too large doses or they may succumb to

some spontaneous disease to which they seem very susceptible during such treatment. We did succeed in getting four animals to the stage where they could be tested against the highly virulent culture and as the treatment of each animal is different from the others a summary of the immunization is given below.

Rabbit 8.—Three injections of a non-virulent strain followed by a mildly virulent strain.

June 6, 1916. Subcutaneous injection of 0.01 cc. of a 24 hour bouillon culture of Hog-cholera Neb. Slight rise in temperature but no loss in weight.

June 20. Subcutaneous injection of 1 cc. of a 24 hour bouillon culture of Hog-cholera Neb. Loss in weight but no increase in temperature.

July 8. Intravenous injection of 0.1 cc. of a 24 hour bouillon culture of Hog-cholera Neb. Rise in temperature but no loss in weight. Another rabbit that had had the same injections died during the night following the intravenous inoculation.

July 21. Subcutaneous injection of 0.1 cc. of a 24 hour bouillon culture of Hog-cholera Ark. No loss in weight and no rise in temperature. Previous tests had shown that this culture in this amount would kill a normal animal in 20 days.

This rabbit was bled three times after the last inoculation and the results of the agglutination tests are given in Table III.

TABLE III.

Agglutination Titer of the Serum of Rabbit 8.

Culture agglutinated.	Limit of agglutination for serum drawn on.		
	Aug. 4	Sept. 19	Oct. 3
Hog-cholera XII	1/80,000	1/51,200	1/3,200
" XII, passage series	1/20,000	1/25,600	1/6,400

Rabbit 9.—Injected with increasing numbers of Hog-cholera Ark. (mildly virulent strain).

June 6, 1916. Subcutaneous injection of 0.0001 cc. of a 24 hour bouillon culture. Rise in temperature but no loss in weight.

June 20. Subcutaneous injection of 0.01 cc. of a 24 hour bouillon culture Rise in temperature and loss in weight extending over a period of 20 days.

July 21. Subcutaneous injection of 0.1 cc. of a 24 hour bouillon culture. Slight loss in weight but no rise in temperature.

This last injection was one that would kill the normal rabbit, so no further injections were given but the animal was allowed to rest until it was tested for immunity to the highly virulent strain. During this period of rest it was bled three times and the limit of agglutination for the hog-cholera bacillus determined. The results of these tests are given in Table IV.

TABLE IV.

Agglutination Titer of the Serum of Rabbit 9.

Culture agglutinated.	Limit of agglutination for serum drawn on.		
	Aug. 4	Sept. 19	Oct. 3
Hog-cholera XII	1/20,000	1/3,200	1/1,600
" XII, passage series	1/5,000	1/1,600	1/1,600

Rabbit 10.—Injected with increasing amounts of Hog-cholera XII (virulent culture).

June 20, 1916. Subcutaneous injection of 0.0000001 cc. of a 24 hour bouillon culture. Rise in temperature but no loss in weight.

July 11. Injection of June 20 repeated. No rise in temperature and no loss of weight.

July 21. Subcutaneous injection of 0.00001 cc. of a 24 hour bouillon culture. No loss in weight or rise in temperature.

Aug. 7. Subcutaneous injection of 0.001 cc. of a 24 hour bouillon culture. No effect on weight or temperature. Control rabbit died in 4 days.

Aug. 19. Subcutaneous injection of 0.01 cc. of a 24 hour bouillon culture. No effect on weight or temperature. The animal was now allowed to rest for some time before its resistance to the highly virulent strain was tested. Table V gives the results of the agglutination tests made during this period.

TABLE V.

Agglutination Titer of the Serum of Rabbit 10.

Culture agglutinated.	Limit of agglutination for serum drawn on.		
	Aug. 31	Sept. 17	Oct. 3
Hog-cholera XII	1/25,600	1/25,600	1/6,400
" XII, passage series	1/25,600	1/25,600	1/6,400

Rabbit 11.—One injection of a sublethal number of Hog-cholera XII bacilli (virulent culture).

Aug. 22, 1916. Subcutaneous injection of 0.00001 cc. of a 24 hour bouillon culture of Hog-cholera XII. Marked loss in weight and rise in temperature. Another rabbit inoculated with the same amount of culture at the same time died in 12 days. In Table VI are given the results of the agglutination tests made on the serum of this rabbit previous to its inoculation with the highly virulent culture.

TABLE VI.

Agglutination Titer of the Serum of Rabbit 11.

Culture agglutinated.	Limit of agglutination for serum drawn on.	
	Sept. 18	Oct. 3
Hog-cholera XII	$\frac{1}{6,400}$	$\frac{1}{3,200}$
" XII, passage series	$\frac{1}{3,200}$	$\frac{1}{3,200}$

On Oct. 3 these four rabbits together with two controls were bled from the ear vein and the sera tested for agglutinins to the highly virulent culture. 3 days later each was given a subcutaneous injection of 0.000001 cc. of a 24 hour bouillon culture of the highly virulent organism in order to test their immunity. The necessary data for the understanding of this test together with the results are given in Table VII. The subcutaneous route was chosen for the inoculation in order to be sure that all the animals received the same amount of culture.

Examination of the table will show that the rabbits previously injected with living cultures were not affected when inoculated with the highly virulent culture, whereas the controls died in 7 days. The agglutination titer of the sera of each of these immune animals was below that found in the serum of the animals injected with heated cultures, yet the latter promptly succumbed to an inoculation with this virulent culture in small amounts.

3 months later two of these immune rabbits were again tested for agglutinins and then injected with a large amount of the highly virulent culture. The results are summarized in Table VIII where it will be seen that one rabbit did not survive this severe test while the animal with the lower titer resisted one thousand times the minimal fatal dose.

TABLE VII.

Test of the Power of Living Cultures to Produce Agglutinins and Immunity.

Rabbit No.	Immunization.				Test of immunity to Hog-cholera XII, passage series, on Oct. 6, 1916.			
	Date of injection.	Strain of hog-cholera.	Amount of 24 hr. bouillon culture injected.	Route.	Time after last injection.	Weight.	Agglutination titer 3 days before test of immunity.	Result of subcutaneous injection of 0.000001 cc. of 24 hr. bouillon culture.
	1916		*cc.*		*days*	*gm.*		
8	June 6	Neb.	0.01	Subcutaneous.	77	3,028	1/6,400	No effect.
	" 20	"	1.0	"				
	July 8	"	0.1	Intravenous.				
	" 21	Ark.	0.1	Subcutaneous.				
9	June 6	Ark.	0.0001	Subcutaneous.	77	3,374	1/1,600	No effect.
	" 20	"	0.01	"				
	July 21	"	0.1	"				
10	June 20	XII	0.0000001	Subcutaneous.	48	2,768	1/6,400	No effect.
	July 11	XII	0.0000001	"				
	" 21	XII	0.00001	"				
	Aug. 7	XII	0.001	"				
	" 19	XII	0.01	"				
11	Aug. 22	XII	0.00001	Subcutaneous.	45	2,024	1/3,200	No effect.
12	Normal animal for control.				0	2,932	0	Died in 7 days. Typical lesions.
13	Normal animal for control; injection one-tenth the amount given other rabbits.				0	2,359	0	Died in 7 days. Typical lesions

The results of these tests clearly indicate that an animal may show agglutinins to the hog-cholera bacillus *in vitro* and yet have no immunity. It cannot be said, however, that these bodies have no relation to immunity for they are present in the sera of all the immune animals though, at the time of the test, not in as high dilutions as in the vaccinated rabbits.

TABLE VIII.

Test of the Power of Living Cultures to Produce Agglutinins and Immunity.

Rabbit No.	Immunization.	Agglutination titer 3 days before test of immunity.	Test of immunity.	
			Amount of 24 hr. bouillon culture injected.	Result.
			cc.	
8	See Table VII.	$\frac{1}{1,280}$	0.0001	Died in 8 days.
11	" " VII.	$\frac{1}{640}$	0.0001	Slight rise in temperature.
6	Control.	0	0.0000001	Died in 9 days.
7	"	0	0.00000001	No effects.

Attempts have been made to differentiate after the method of Joos (8) the agglutinins in the vaccinated rabbit from those found in the immune animals. No difference has been found in the susceptibility to heat of the agglutinins from these two sets of animals nor do the sera act differently on heated bacteria.

Intra Vitam Agglutination.

The work of Bull (5) suggested the comparison of the *intra vitam* agglutination in vaccinated rabbits and in those immunized by the use of living cultures. When a suspension of the living organisms from the highly virulent strain are injected intravenously into either of these animals they are promptly clumped and rapidly disappear from the circulation. Films made from the liver half an hour after the injection show cells packed with bacteria. Most of the phagocytic cells found were polymorphonuclear leukocytes but a few phagocytic endothelial cells were also present. No difference was noted in the reactions of animals from the two sets though one was immune and the other was very susceptible to the bacterium injected. The most interesting phase of this work was that the control animals also showed typical *intra vitam* clumping. This fact was verified repeatedly but only one experiment will be given.

The growth from two 24 hour agar slants inoculated with Hog-cholera XII passage virus was suspended in 15 cc. of salt solution. Shaken and centrifugal-

ized for ½ hour. Removed the supernatant fluid and suspended the residue in 5 cc. of salt solution and shook vigorously to break up clumps. Injected intravenously into a normal rabbit weighing 2,270 gm. Blood removed from the heart at stated intervals and dilutions made for plate cultures. The first dilution was made in a glass-stoppered bottle and was shaken for some time to break up clumps. At the same time films were made that later were stained with Manson's methylene blue. The findings in the films as well as the results of the plate counts are given in Table IX.

40 minutes after the injection, the rabbit was chloroformed and films were made from the liver. Cells containing bacteria were present but were not numerous.

TABLE IX.

Intra Vitam Agglutination Test Using a Normal Rabbit.

Time after injection.		Bacteria per cc. in heart's blood.	Result of examination of film.
min.	*sec.*		
0	31	30,000,000	Large clumps of bacteria embedded in a blue-staining homogeneous mass.
2	30	4,800,000	No bacteria found.
5	24	5,950,000	" " "
15	0	75,000	" " "
30	0	117,000	" " "

This experiment, as well as the others made, shows that in the normal rabbit there is a prompt clumping of the injected bacteria, a rapid disappearance from the blood stream as shown by films and plate counts, and a phagocytosis of the bacteria by cells in the liver and in other organs. The centrifugalization of the bacteria had nothing to do with the clumping for it occurred in other animals where a suspension made directly from the agar slant was injected. When the dilutions of blood were not shaken in glass-stoppered bottles, plates made 30 minutes after the injection and containing $\frac{1}{500}$ cc. of blood were sterile.

SUMMARY AND CONCLUSION.

Rabbits may show a high agglutination titer to the hog-cholera bacillus and have no immunity and on the other hand immune animals may have a comparatively low agglutination titer. In other words, with this organism the height of the agglutination titer does

not indicate the degree of immunity. As this bacillus so closely resembles the typhoid bacillus biologically and pathologically, it seems safe to conclude, until evidence is brought forth to the contrary, that in man the height of the agglutination titer does not indicate the actual degree of immunity to the latter organism. The same would apply to other members of the typhoid-colon group. It would not be wise to draw a more general conclusion until other organisms have been tested. This does not mean that agglutinins are not related to immunity but it brings up the question of the wisdom of using them as a guide in immunization with the colon-typhoid group.

When injected into the normal, vaccinated, or immune rabbit, the virulent hog-cholera bacillus is rapidly clumped and disappears from the circulation. 40 minutes after injection these organisms can be found in phagocytes in the liver. The fact that the normal rabbit gives this *intra vitam* agglutination is an exception to the findings of Bull that virulent organisms remain in the circulation for some time after injection.

BIBLIOGRAPHY.

1. TenBroeck, C., *J. Exp. Med.*, 1917, xxvi, 437.
2. Smith, T., and Moore, V. A., *U. S. Dept. Agric., Bureau Animal Industry, Bull. 6*, 1894, 41.
3. Smith, T., and Reagh, A. L., *J. Med. Research*, 1903, ix, 240.
4. Shoukévitch, J., *Ann. Inst. Pasteur*, 1910, xxiv, 728.
5. Bull, C. G., *J. Exp. Med.*, 1916, xxiv, 25.
6. Smith, T., *U. S. Dept. Agric., Ann. Rep., Bureau Animal Industry*, 1886, 38.
7. TenBroeck, *J. Exp. Med.*, 1916, xxiv, 213.
8. Joos, A., *Centr. Bakteriol., Ite Abt., Orig.*, 1903, xxxiii, 762.